

EFFECT OF ORAL HEPARIN ON HOMOCYSTEINE INDUCED CHANGES IN HEMODYNAMIC PARAMETERS AND OXIDATIVE STRESS

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ABSTRACT

Several studies have found a positive correlation between hypertension and hyperhomocysteinemia. Increasing evidence implicates oxidative stress as one of the initiating events closely linked to the homocysteine's ability to damage endothelium, subsequently causing vascular dysfunction. We previously found that heparin protects cultured endothelial cells from free radical injury and oral heparin at 1 mg/kg/48h prevents venous thrombosis in a rat model *in vivo*. Our objective was to study the protective effects of oral heparin in a rat model with elevated plasma homocysteine (Hcy) concentrations, and begin to elucidate whether the pathophysiological effects of Hcy are mediated through an oxidative mechanism causing endothelial dysfunction.

Elevated plasma Hcy levels were induced by feeding male Wistar Kyoto rats a diet containing an additional 1.7% methionine for 8 weeks. Groups included rats fed additional methionine, methionine plus oral heparin (1 mg/kg/48h by gastric feeding tube), and age-matched controls fed normal rat chow. At the end of 8 weeks of treatment, rats were anesthetized using 1.5% isoflurane in 100% oxygen. Hemodynamics parameters were assessed by inserting a Millar Mikro Tip pressure transducer into the left ventricular chamber and the thoracic aorta. Fasting plasma total Hcy levels were measured using a Hcy immunoassay kit with an Abbott IM_x instrument. Malondialdehyde (MDA) concentrations, a lipid peroxidation product and marker for oxidative stress, was measured by a spectrophotometric method in serum and tissue samples. Glutathione (GSH) concentrations, an important antioxidant for low-level oxidative stress was measured by HPLC in plasma and tissues samples. Lastly, tissue samples from each experimental group were stained with the TUNEL method to assess their respective percentage of apoptotic endothelial cells. Results were expressed as mean \pm S.E. Unpaired Student's two-tailed *t*-test was employed to assess the difference between groups with $p < 0.05$ considered significant.

Plasma Hcy was significantly elevated after 8 weeks in the methionine (7.17 ± 0.46 $\mu\text{mol/L}$) and methionine plus heparin treated rats (7.02 ± 0.40 $\mu\text{mol/L}$) compared to control (5.46 ± 0.36 $\mu\text{mol/L}$). All measures of arterial pressure, systolic (SP) and diastolic pressure (DP) and mean arterial pressure (MAP), were significantly elevated in rats fed the methionine diet without

heparin (119.9 ± 3.9 mmHg; 90.3 ± 3.5 mmHg; 97.7 ± 2.9 mmHg, respectively) compared to controls (107.8 ± 2.5 mmHg; 79.2 ± 2.1 mmHg; 88.8 ± 2.2 mmHg, respectively) but not compared to heparin (114.7 ± 3.3 mmHg; 83.4 ± 2.4 mmHg; 93.8 ± 2.7 mmHg, respectively). Left ventricular end diastolic pressure (LVEDP) was significantly elevated with the methionine diet without heparin (14.2 ± 2.5 mmHg) but not with heparin treatment (8.4 ± 1.9 mmHg) versus controls (7.1 ± 1.1 mmHg). Also, left ventricular systolic pressure (LVSP) was significantly elevated in the methionine fed rats after 8 weeks (122.6 ± 3.2 mmHg) compared to controls (112.3 ± 2.9 mmHg). Heparin treatment had no effect on LVSP (119.9 ± 3.2 mmHg).

Additionally, the results of this study showed that oral heparin treatment significantly decreased liver MDA concentrations (2.42 ± 0.28 nmol/mg protein) compared to the methionine treated group (5.10 ± 0.96 nmol/mg protein) and methionine treatment alone significantly reduced MDA concentrations in kidney tissue (1.59 ± 0.12 nmol/mg protein) compared with controls (3.26 ± 0.66 nmol/mg protein). Methionine diet significantly decreased GSH concentrations in plasma (0.59 ± 0.59 μ mol/L) compared with controls (4.24 ± 0.94 μ mol/L) and oral heparin treatment significantly attenuated the decrease in GSH concentrations in left ventricle tissue samples (0.0229 ± 0.0023 μ mol/mg protein) compared with methionine treatment alone (0.0135 ± 0.0016 μ mol/mg protein).

Elevated plasma homocysteine levels, induced by methionine diet feeding significantly increased the percent of apoptotic endothelial cells in the aortas ($17.04 \pm 3.74\%$) and superior mesenteric arteries ($17.99 \pm 1.90\%$) of WKY rats compared with control aortas and mesenteric arteries ($6.08 \pm 3.24\%$; $7.43 \pm 1.62\%$, respectively) and compared to oral heparin treated mesenteric arteries ($7.31 \pm 1.18\%$).

The results of this study showed that elevated plasma levels of Hcy correlate with the development of hypertension, defined as significantly increased arterial pressure. Oral heparin treatment prevented the significant increase in arterial pressures and LVEDP, decreased MDA concentrations and therefore the oxidative stress on the liver, attenuated the decrease caused by elevated plasma Hcy in left ventricle GSH concentrations, and significantly reduced the number of apoptotic endothelial cells in the superior mesenteric artery of high methionine fed rats. We conclude that elevated levels of plasma Hcy contributes to the development of hypertension and furthermore towards the onset of heart failure likely through an oxidative mechanism and that

oral heparin reduces the overall oxidative stress in specific physiological environments, preventing Hcy mediated endothelial cell apoptosis.

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LIST OF ABBREVIATIONS

- dP/dt	myocardial index of ventricular relaxation
+ dP/dt	myocardial index of ventricular contraction
$^1\text{O}_2$	Singlet oxygen
ACE	Angiotensin-converting enzyme
ACh	Acetylcholine
ACTH	Adrenocorticotrophic hormone
ADH	Anti-diuretic hormone
ADMA	Asymmetric dimethylarginine
ALA	Alpha-lipoic acid
Ang II	Angiotensin II
ANP	Atrial natriuretic peptide
ANS	Autonomic nervous system
ATP	Adenosine triphosphate
AV	Atrioventricular
BNP	Brain natriuretic peptide
BP	Blood pressure
Ca^{2+}	Calcium ion
CAD	Coronary artery disease
CBS	Cystathionine- β -synthase
CKD	Chronic kidney disease
CNS	Central nervous system
CO	Cardiac output
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
Cu-Zn-SOD	Copper-Zinc-superoxide dismutase
CV	Cardiovascular
CVD	Cardiovascular disease
ddH ₂ O	Double distilled water
DNA	Deoxyribonucleic acid

DP	Diastolic pressure
DTNB	5,5'-dithiobis-2-nitrobenzoic acid
DTT	Dithiothreitol
EC	Endothelial cell
ECF	Extracellular fluid
EC-SOD	Extracellular superoxide dismutase
EDP	End diastolic pressure
EDTA	Ethylene diamine tetra acetic acid
EDV	End diastolic volume
eNOS	Endothelial nitric oxide synthase
EPO	Erythropoietin
ESV	End systolic volume
ET-1	Endothelin-1
FGF	Fibroblast growth factor
GAG	Glycosaminoglycans
GPx	Glutathione peroxidase
GSH	Glutathione
GSSG	Glutathione disulfide
H ₂ O ₂	Hydrogen peroxide
HAc	Glacial acetic acid
HB-EGF	Heparin-binding epidermal growth factor
Hcy	Homocysteine
hHcy	Hyperhomocysteinemia
HOCl	Hypochlorous acid
HPLC	High performance liquid chromatography
HR	Heart rate
HS	Heparan sulfate
HSPGs	Heparan sulfate proteoglycans
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IR	Insulin resistance

K ⁺	Potassium ion
LBW	Low birth weight
LDL	Low-density lipoproteins
LMWH	Low molecular weight heparins
LV	Left ventricle
LVDP	Left ventricular developed pressure
LVEDP	Left ventricular end-diastolic pressure
LVH	Left ventricular hypertrophy
LVSP	Left ventricular systolic pressure
MAOIs	Monoamine oxidase inhibitors
MAP	Mean arterial pressure
MD	High methionine diet
MDA	Malondialdehyde
MI	Myocardial infarction
MMP	Matrix metalloproteinases
Mn-SOD	Manganese-superoxide dimutase
MPO	Myeloperoxidase
MRP	Multidrug resistance protein
MTHFR	Methylenetetrahydrofolate reductase
MVP	Mean venous pressure
Na ⁺	Sodium ion
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NO ₂ ⁻	Nitric dioxide
Nox	NADPH oxidases
NSAIDs	Non-steroidal anti-inflammatories
O ₂	Molecular oxygen
O ₂ ⁻	Superoxide radical
OH [·]	Hydroxyl radical
ONOO ⁻	Peroxynitrite
OSAH	Obstructive sleep apnea/hypopnea

PI	Propidium iodide
PNS	Parasympathetic nervous system
PP	Pulse pressure
RAS	Renin-angiotensin system
RBC	Red blood cells
ROO [•]	Peroxyl radical
ROS	Reactive oxygen species
SA	Sinoatrial
SAM	S-Adenosyl methionine
SAH	S-Adenosyl Homocysteine
SD	Sprague Dawley
SDS	Sodium dodecyl sulfate
SMC	Smooth muscle cells
SNS	Sympathetic nervous system
SOD	Superoxide dismutase
SP	Systolic pressure
SV	Stroke volume
TBA	Thiobarbituric acid
TEP	Tetra ethoxy propane
TGF	Tissue growth factor
tHcy	Total homocysteine
t-PA	Tissue plasminogen activators
TPR	Total peripheral resistance
Tx	Triton-X-100
UV	Ultraviolet
VSM	Vascular smooth muscle
WKY	Wistar Kyoto
XO	Xanthine oxidase

1.0. REVIEW OF THE LITERATURE

1.1. INTRODUCTION

1.1.1. Blood Pressure

Blood pressure (BP) is defined as the hydrostatic force of blood that exerts pressure against the walls of a blood vessel (Kablund, 2005). Blood pressure is dynamically regulated and fundamentally required for life. Arterial BP is pulsatile, in rhythm with the cardiac cycle. Arterial BP is expressed as systolic pressure which occurs during heart contraction and diastolic pressure during heart relaxation. Mean arterial pressure (MAP) is considered to be the perfusion pressure that supplies blood to organ systems. The MAP is not the arithmetic mean between systolic and diastolic pulse pressures. It is the sum of the diastolic pressure and a third of the pulse pressure, which is equal to the systolic minus the diastolic pressure.

$$MAP = DP + \frac{1}{3}(SP - DP)$$

(1.1)

Blood pressure can be understood by Ohm's law, in which the change in BP in a vessel is equal to the product of blood flow and vessel resistance. As blood flow is typically laminar and is contained within a closed system, flow is related to cardiac output (CO). For any given CO, the pressure generated by ventricular contraction is subject to resistance along its travel pathway. This is known as vascular resistance. Total peripheral resistance (TPR) is the total vascular resistance in the systemic circulation, equal to the Mean Arterial Pressure (MAP) minus the Mean Venous Pressure (MVP) divided by CO.

$$TPR = (MAP - MVP)/CO \quad (1.2)$$

The cardiovascular (CV) system intrinsically includes the heart and the vasculature, therefore defining arterial BP as the product of CO times total peripheral resistance (TPR).

$$BP = CO \times TPR \quad (1.3)$$

1.1.1.1. Cardiac Output

Cardiac output is the volume of blood ejected from the heart per unit of time. Cardiac output on the left side of the heart is directly proportional to the volume ejected from and pressures generated by the left ventricle (LV). This relationship is the same for the right side of the heart. Equal CO from both the right and left sides of the heart is an indicator of CV health such that the stroke volume (SV) of blood entering the lungs for oxygenation is equal to the SV of blood ejected for tissue perfusion. As per the formula for BP, increases in CO for any given TPR will increase BP, and vice versa. The mathematical relationship for CO is the product of SV, the volume of blood ejected from one ventricle with each contraction, and heart rate (HR) the number of heart contractions per minute

$$CO = SV * HR \quad (1.4)$$

1.1.1.2. Heart Rate

The sinoatrial (SA) node is the heart's pacemaker, initiating myocardial contraction and when functioning properly sets a HR in a healthy, relaxed adult at approximately 60 - 80 beats/min (Thorin & Thorin-Trescases, 2009). Heart rate is dynamic and changes in relation to body system demands. The autonomic nervous system predominantly controls the regulation of HR.

1.1.1.2.1. Autonomic Nervous System (ANS) Regulation

The CV centre in the medulla oblongata is the epicenter for CV regulation. The CV centre is capable of increasing or decreasing neural input to the SA node, atrioventricular (AV) node and myocardium, slowing or increasing the HR and influencing contractility. However, autonomic innervation does not set or undo the fundamental HR rhythm.

Parasympathetic innervations that regulate HR are carried by cranial nerve X (Vagus nerve). Axons from the vagus nerve terminate in the SA node, the AV node and atrial myocardium. Acetylcholine is released from these axons, slowing the HR. Since very few vagus axons terminate on the ventricles, this type of autonomic regulation likely has little effect on ventricular contractility.

Sympathetic neurons, known as the cardiac accelerator nerves, extend out to the SA node, AV node and most of the myocardium. Cardiac accelerator nerves release norepinephrine, and

both norepinephrine and epinephrine are released from the adrenal medulla into the circulation, both which increase the rate of spontaneous depolarization in the SA and AV nodes, increasing HR. When either is bound to receptors on the myocardium, calcium ion cytosolic entry increases, enhancing contraction. It is important to note that norepinephrine and epinephrine have different effects on HR and contractility dependent upon which receptor type they're bound to. Alpha (α) adrenergic receptors predominantly trigger vascular smooth muscle contraction, which has no direct effect on HR or contractility (Wray, Nishiyama, & Richardson, 2009). Beta (β) adrenergic receptors increase myocardium contraction through β_1 receptor activation. Norepinephrine or epinephrine are defined with respect to β -adrenergic receptors, as positive chronotropic agents, increasing HR, and positive inotropic agents, increasing contractility (Chen-Izu, et al., 2000).

1.1.1.3. Stroke Volume

The heart's primary function is a pump for blood, not a reservoir. Blood that enters the heart is intended to be ejected within a few cardiac cycles to ensure continuous delivery of oxygenated blood to organs and tissues. Stroke volume is defined as the volume of blood ejected from one or the other but not both ventricles during each contraction. The three major factors that regulate SV are preload, afterload and contractility.

1.1.1.3.1. Preload

Preload is the amount of stretch placed onto the ventricular wall during diastole (relaxation) while it passively fills with blood from the atria before and after atrial contraction. Ventricular end diastolic volume (EDV) and end-diastolic pressure (EDP) is therefore proportional to preload. An increase in the length-tension relationship of the myocardium, implies that the further the ventricle is stretched during filling, the greater the muscle length available for contraction, resulting in a larger potential force or pressure generated during ventricular systole. This also means that the more the ventricle is filled during diastole, the greater the SV will be during systole. This relationship is defined by ventricular pressure-volume loops and the Frank-Starling Law of the Heart. Preload is determined by venous return, atrial systolic pressures, HR and SV (Greenway & Lautt, 1986).

1.1.1.3.1.1. Heart Rate

Heart rate significantly affects the time available for ventricular diastole. As the HR increases, so does the rate of blood ejection from the ventricles and the time allowed for

ventricular filling. A shorter duration for filling corresponds to a lower EDV. A lower EDV in response to increased HR is equal to decreased preload, changing the hemodynamics of the heart with respect to decreased ventricular systolic pressure and SV as per Frank-Starling's Law.

1.1.1.3.2. Afterload

Afterload is defined as the pressure achieved in the LV at maximum systolic elastance or stretch (Greenway & Loutt, 1986). Changes in afterload are determined by TPR, aortic compliance, SV and by other factors causing impedance to blood flow (Greenway & Loutt, 1986). The pressure generated by the ventricles during systole must be higher than aortic pressure, as required to open the aortic valve and permit the ejection of blood. Afterload is the force the heart has to overcome to eject blood, which with respect to pressure-volume loops, correspondingly increases pressures generated by the ventricle during systole. However, despite this increase in ventricular systolic pressure, there is an overall decrease in SV and therefore an increase in end-systolic volume (ESV). A decrease in SV is equal to a reduction in the ejection fraction of the ventricle, causing a subsequent increase in preload in the next contraction, as per the Frank-Starling Law.

1.1.1.3.3. Contractility

Contractility, the strength of contraction at any preload is the third factor that influences SV. End diastolic volume or preload is the volume that fills the ventricle during ventricular diastole, and the ESV is the volume remaining in the ventricle after contraction. Inotropic agents will increase (positive inotropy) or decrease (negative inotropy) the contraction of the ventricle, affecting the ejection of blood. The ventricles normally retain a small volume of blood after contraction. The force of ventricular contraction or amount of pressure generated by the myocardium can reduce or increase the end systolic volume.

1.1.1.3.3.1. Inotropic Agents

Positive inotropic agents such as norepinephrine and epinephrine, acting on β_1 -adrenergic receptors increase ventricular contractility, where given no change in preload, more powerful contractile forces generated in the ventricles are associated with more complete emptying. Maximum emptying of the ventricle would reduce ESV, which usually relates to a higher CO.

All β -adrenergic agonists will exert a positive inotropic effect on the heart increasing both the force and velocity of contraction. As well positive inotropic agents such as cardiac glycosides or digoxin act independently of adrenergic catecholamine activities. Side effects of

prolonged positive inotropy or overdose of a positive inotropic agent include anorexia, nausea, and confusion, along with a HR below 60 beats per minute (Colucci, Wright, & Braunwald, 1986).

Negative inotropic agents such as verapamil, a myocardium selective calcium channel blocker drug, significantly reduce CO (Kondo & Shibata, 1984). As this effect may be coupled with effects on smooth muscle in vessels and vasodilation, which would decrease TPR, the overall effect would be a measureable drop in BP. The side effects of these medications are similar to hypotensive symptoms such as dizziness, headache and constipation (Hoffmeister, Hepp, & Seipel, 1987).

1.1.1.3.3.2. Myocardial Indices of Contractility and Relaxation

Indices that provide information about the rate of contractility and relaxation of the myocardium are useful when describing the behavior of cardiac muscle fibers during the cardiac cycle. The index of myocardial contractility and relaxation is the change in ventricular pressure over time, $+ dP/dt$ or $- dP/dt$ (P: pressure; t:time; d: delta/change) respectively. These indices may also be considered the rate of contractility and relaxation over time. The rate of change in ventricular pressure during systole indicates myocardial responsiveness to contractile signals. Conversely, the rate of change in ventricular pressures immediately following ventricular systole, indicates how quickly isovolumetric relaxation takes place before ventricular filling next occurs.

1.1.1.3.3.3. Venous Return

The venous system is comprised of a network of highly collapsible and distensible blood vessels which transport deoxygenated blood under low pressure back to the heart from the extremities. The venous system is called a capacitance system, as it acts as a reservoir for approximately 64% of total blood volume. Venous return to the right atrium is driven by pressure gradients. Most of the driving pressure exerted by cardiac contraction is lost by the time blood reaches the venous system, due mainly to the high resistance passage of blood through the arterioles. The pressure in the right atrium is considered to be 0 mmHg, the residual pressure in the venous system (approximately 17 mmHg) is enough of a driving force to pump blood into the heart for re-circulation (Guyton, et al., 1957). There are several factors which facilitate venous return including a cardiac-suction event, decreased pressure in the right atrium

and increases in venous pressures from the skeletal muscle pump which increase venous driving pressure gradients towards the heart. Increased venous return increases SV, and thus CO.

1.1.1.4. Total Peripheral Resistance

The total peripheral resistance of the systemic circulation is the sum of all the resistances exerted by the systemic vasculature. Vascular resistance opposes blood flow because of frictional forces between blood and the walls of the blood vessels. The formula for BP indicates that increases in TPR will result in increased BP for a given CO. Three factors contributing to blood flow resistance are explained by Poiseuille's Law and the following equation:

$$R \propto \frac{\eta * L}{r^4} \quad (1.5)$$

The equation describes how resistance to flow (R) is directly proportional to blood viscosity (η), vessel length (L) and internal vessel radius (r) (Sutera & Skalak, 1993). Notably, the vessel radius is the most influential factor contributing to the resistance to flow.

1.1.1.4.1. Blood Viscosity

Blood viscosity may contribute to resistance to blood flow. The major components of blood influencing viscosity are red and white blood cells, platelets and plasma proteins. The viscosity of normal blood is approximately three times greater than water. Changes to the normal composition of the blood, such as increased red blood cells (hematocrit) will correspondingly increase viscosity. As the hematocrit rises to levels of 60 or 70%, from normal values of 38 to 48%, blood viscosity increases by two to three times normal, causing significant resistance to its flow (Lenz, C. et al., 2008) (Purves, et al., 2004). Increases to hematocrit are common in disorders such as polycythemia (Tefferi, 2003). Conversely, anemia, low red blood cell percent can decrease blood viscosity, which has the potential to lead to heart failure due to a significant drop in BP and reduced blood flow and oxygen delivery to organ systems (Iyengar & Abraham, 2005). Although, viscosity is a factor in the resistance of blood flow, outside of disease and illness, it is not a significant contributor to TPR.

1.1.1.4.2. Vessel Length

Resistance to blood flow increases with increasing vessel length as per Poiseuille's Law. Considering the organization of the vascular tree, the length and radius of different vessels are responsible for the marked drop in BP seen as blood flows from the heart to the extremities. Microvascular rarefaction is classified as the disappearance of microvessels, including the

arterioles and capillaries. The disappearance of vessels effectively decreases the length of resistance to blood flow. Decreasing vessel length should correspondingly decrease TPR. However the opposite is true as the resistance to blood flow increases as there are fewer pathways available for the same volume of blood. This idea has been observed in several studies, showing that microvascular rarefaction, increases TPR and the overall pattern of blood flow distribution in experimental animals which develop hypertension (Greene, et al., 1989). Vessel length is not considered a significant contributor to TPR.

1.1.1.4.3. Vessel Radius

Vessel radius is the most significant factor affecting the resistance to flow. This dynamic can be understood by Poiseuille's Law, since resistance is inversely proportional to the 4th power of the radius. Smaller lumen radius increases the friction between blood and the walls of the vessel, resulting in an overall increase in resistance to flow and correspondingly an increase in BP. The lumen of the aorta is relatively wide (1.25 cm radius) and highly elastic to absorb the high pressures generated by the LV during systole (Lang, et al., 1994). Decreasing lumen size, restricts blood flow from the aorta to the large arterial branches (0.2 cm radius). Flow is again opposed when blood travels to the arterioles (30 μ m radius) and again to the capillaries (3.5 μ m radius) (Zamir, 1977). But given the large number of capillaries in comparison to arterioles, the velocity of blood flow slows, however the resistance to flow does not increase proportionally.

The cross-sectional area of each type of vessel is inversely related to the velocity of blood flow. The cross-sectional area of a blood vessel increases with each branching of that vessel, meaning that blood flow velocity decreases where cross-sectional area is the greatest. The velocity of blood flow is the slowest in the capillaries. Blood vessel length and radius both contribute to cross-sectional area. Zamir (1977) showed that there is greater than a 1000 fold increase in cross-sectional area between the aorta and the capillaries, 3 – 5 cm² compared to 4500 – 6000 cm² respectively. Ultimately, because of their highly muscularized structure, arterioles are able to significantly alter their vessel radius, and are therefore considered the major resistance vessel, and determinant of TPR, making vascular smooth muscle contractility an important factor in BP control.

1.1.1.4.3.1. Vasoconstriction and Vasodilation

The most significant resistance vessels in the vascular tree are the arterioles, which have highly muscular, well-innervated walls. Relative to the low resistance of the arteries, arteriole

radii resistance is responsible for reducing mean arterial BP from 93 mmHg to approximately 37 mmHg prior to entering the capillaries (Zamir, 1977). The radii of arterioles are susceptible to both vasoconstriction, vessel narrowing, and vasodilation, vessel widening. This function allows the arterioles to modulate vascular tone and blood flow resistance by utilizing their precapillary sphincters to variably distribute blood flow to various organ systems depending upon metabolic needs and regulate arterial BPs.

1.1.2. Blood Pressure Regulation

Various organ systems play a role in the regulation of CO and TPR and therefore BP. In Figure 1 below, several major feedback loops highlight the importance of CO or TPR in regulation of arterial pressure. The feedback loops will be discussed as follows: (1) baroreceptors located in the aortic arch and the carotid sinus can detect overall changes in arterial pressures and respond accordingly through the ANS by activating either its sympathetic or parasympathetic divisions, (2) the chemoreceptor system of the peripheral nervous system will respond to ischemia when BP falls and act through the sympathetic nervous system (SNS) to control arterial pressure, (3) ischemia detected by chemoreceptors in the central nervous system (CNS) will respond through the SNS to control arterial pressure, (4) the renal-fluid output system acts through changes in total body volume to control arterial pressure, (5) the anti-diuretic hormone (ADH) and thirst control centre cause changes in blood volume to regulate BP, (6) aldosterone also acts by changing blood volume to control arterial pressure, (7) the renin-angiotensin system controls TPR to induce changes to arterial pressure, (8) capillary pressure and filtration system acts by redistributing body fluid between the blood and interstitial fluid to help control BP, (9) the volume of blood in the vasculature acts through changes in venous capacitance to alter venous return and CO and therefore arterial pressure.

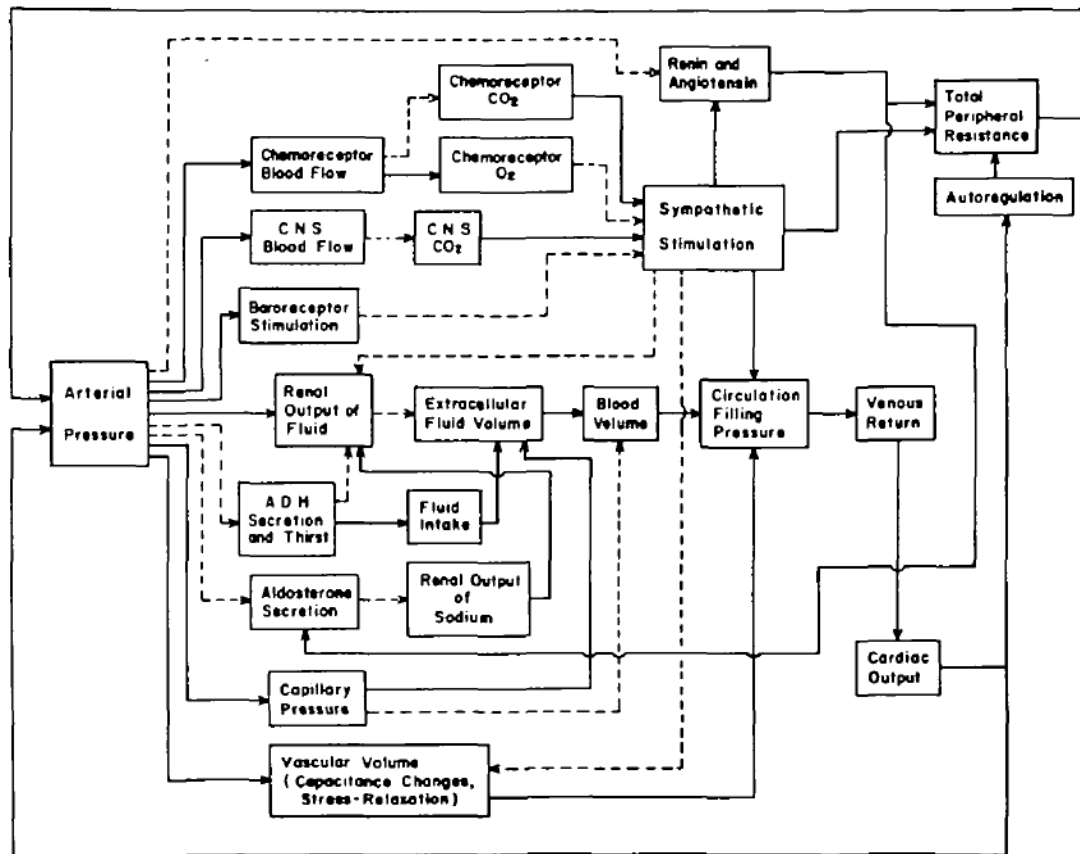


FIGURE 1

Block diagram showing the principal factors in arterial blood pressure control. The broken lines represent negative effects. CNS = central nervous system and ADH = antidiuretic hormone.

Figure 1 Factors of arterial blood pressure control. (Guyton, et al., 1974), with Permission from Wolters Kluwer Health

1.1.2.1. Baroreceptors

Located within the carotid sinus and in the aortic arch, baroreceptors respond quickly to changes in BP. They are stretch-sensitive mechanoreceptors which transmit neural impulses, firing most frequently in response to increased stretch/BP. The negative feedback response system utilizes both the SNS through innervations to the heart and vasculature and the parasympathetic nervous system (PNS) via the vagus nerve. Reduced stretch of the baroreceptors increases SNS stimulation and decreases PNS stimulation, causing increased TPR and CO via: (1) increasing the contractility of the heart through β_1 -adrenergic receptor stimulation; (2) increases HR via the activation of cardiac accelerator nerves in the medulla and (3) causes vasoconstriction through the activation of α_1 -adrenergic receptors. When BP is raised

back to its homeostatic set-point the cycle is reversed activating the PNS and reducing the SNS, resulting in vagus nerve mediated decrease to HR, decreasing both CO and BP. In hypertension, baroreceptor resetting is commonly incomplete (Lohmeier, 2001). Additionally, baroreceptors have been shown to suppress renal sympathetic nerve activity which promotes sodium excretion and further BP compensation in hypertension (Malpas & Barrett, 2002).

1.1.2.2. Peripheral Chemoreceptors

Peripheral chemoreceptors located in the carotid and aortic bodies and also preganglionic chemoreceptors in the thorax and abdomen, are essential for detecting changes in the partial pressure of arterial blood oxygen and for initiating homeostatic reflexes which are critical for responding to hypoxia. Chemoreceptors are sensitive and respond to changes in blood oxygen and carbon dioxide concentrations/partial pressures and blood pH. Low blood oxygen partial pressures can result from a myriad of physiological and pathological changes. Regardless of the initiating change, a decrease in blood oxygen results in tissue ischemia causing tissue death and dysfunction. Interestingly, Fukuda et al. (1987) demonstrated that spontaneously hypertensive rats have an enhanced chemoreceptor response to hypoxia as compared with normotensive rats. Furthermore, Habeck (1991) reported that only patients with essential hypertension and not renal hypertension exhibit the same increase in receptor response. Three different SNS actions which increase BP are intrinsic to the chemoreceptor response to low blood oxygen or high carbon dioxide partial pressures: (1) stimulation of respiratory centers in the medulla oblongata which increases pulmonary ventilation, reciprocating an increase in venous return and CO, modestly increasing BP; (2) increased TPR via SNS activation of α_1 -adrenergic receptors and lastly (3) mild tachycardia and increases in contractility caused by SNS β_1 -adrenergic receptor stimulation.

1.1.2.3. CNS Chemoreceptors

Blood flow to the CNS is essential for life. As such, chemoreceptor responses to CNS ischemia are fast. Centrally located chemoreceptors in the CNS respond primarily to increases in the partial pressure of carbon dioxide, or hypercapnia. When these receptors detect hypercapnia, the vasomotor area of the medulla oblongata is stimulated sending nerve impulses through the spinal cord directly onto the walls of resistance vessels causing vasoconstriction, elevating TPR and BP. Guyenet et al. (2010) also indicate that the SNS response to hypercapnia includes a

wake-promoting and interoceptive awareness in awake individuals elicited through further noradrenergic and serotonergic influences.

1.1.2.4. Renal Fluid Output

Changes in blood fluid volume have a profound effect on BP as it is directly proportional to the amount of fluid exerting pressure on blood vessel walls. The renal system is the most important regulator of body fluid volumes. This type of BP regulation is a relatively slow mechanism for BP control. Increases in blood hydrostatic pressure, filters water from the blood through the glomerulus into the renal tubules and eventually into the collecting duct for elimination from the body. Patients are diagnosed with acute renal failure when this system fails, marked by decreased urine output, hypertension and increased mortality (Liangos et al., 2005). The most effective renal regulation is accomplished by the secretion and actions of anti-diuretic hormone (ADH) and aldosterone.

1.1.2.5. Anti-diuretic Hormone (ADH)

Anti-diuretic hormone is secreted in response to lowered BP and compensates by increasing blood volume and to some extent TPR. Osmoreceptors detect changes in the osmolarity of blood, the relative ratio of plasma proteins in blood, that when elevated, indicate low blood volume stimulating the release of ADH from the posterior pituitary gland. ADH acts in three different ways to increase BP: (1) controls the re-absorption of water and urea in the distal tubules and collecting duct, increasing the amount of water in the blood while concentrating and decreasing urine volumes; (2) stimulates thirst and therefore increases fluid intake and correspondingly fluid volume; and (3) causes vasoconstriction through arginine vasopressin receptor responses which have been shown to produce endothelin and increase vascular smooth muscle intracellular Ca^{2+} concentrations (Ikeda, Kohno, & Takeda, 1995).

1.1.2.6. Aldosterone

Aldosterone is secreted from the cortex of the adrenal gland and regulates BP by increasing blood volume through the renal system. Its overall effect is to increase the reabsorption of Na^+ and water in the distal tubules and collecting duct of the kidney and promote the secretion of K^+ ultimately increasing BP via changes in overall blood volume. Various neural, hormonal and ionic signals trigger aldosterone release from the adrenal cortex. Aldosterone secretion is primarily driven by K^+ plasma concentrations, which are believed to cause membrane depolarization opening voltage-gated channels in the zona glomerulosa of the

adrenal gland (Boukroun, et al., 2010). Angiotensin II (Ang II) is another primary factor of aldosterone release, acting directly on the adrenal cortex (Williams & Dluhy, 1972). Furthermore, Ang II increases the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary which in turn promotes aldosterone synthesis (Brown, Strott, & Liddle, 1972). When water and sodium are reabsorbed in the kidney tubules, circulating volumes increase appearing as increased juxtaglomerular apparatus perfusion. This type of detection by the juxtaglomerulus apparatus inhibits further renin release from the kidneys shutting down the renin-angiotensin system and also further aldosterone release. Baroreceptor have also been implicated as an inhibitor of aldosterone secretion ultimately via ACTH inhibition (Giannattasio, et al., 1989). Lastly, again through the renin-angiotensin systems (RAS), the SNS also modulates aldosterone release.

1.1.2.7. Renin-angiotensin System (RAS)

The primary responses of the RAS are to cause arteriolar vasoconstriction, thus increasing TPR, and reabsorbing sodium and water thus increasing blood volume. The system is primarily activated by a decline in arterial BP. The main bioactive agent of the system is Ang II, which is formed through a cascade of biochemical events initiated by the release of renin from the kidneys. When perfusion to the juxtaglomerular apparatus decreases the kidneys release renin. Angiotensinogen is synthesized in the liver and is converted into Angiotensin I through the renin dependent action on angiotensinogen. Angiotensin II is then converted from angiotensin I by angiotensin-converting enzyme (ACE) located on endothelial cells, primarily in lung capillaries. Ang II is a potent vasoconstrictor of arterioles by potentiating the release of norepinephrine on post-ganglionic adrenergic fibers, amplifying α -adrenoreceptor-mediated vasoconstriction (Mancia, et al., 2006). As previously discussed, Ang II regulates the release of aldosterone and ADH, and is therefore indirectly responsible for promoting water resorption in the kidney.

1.1.2.8. Capillary Pressure

Blood pressure increases are reflected throughout the vascular system such that increased arterial pressure will also cause an increase in capillary pressures. The hydrostatic pressure of blood inside the capillaries forces water into interstitial spaces defined by the Starling equation which defines the forces across the semipermeable wall of the capillaries. Filtration through the capillaries can be markedly increased by immune mediated release of vasoactive agents, such as

histamine, that increase its relative permeability, and changes to capillary and interstitial oncotic pressure and lymphatic flow. Edema, is a symptom of excessive capillary filtration, where large amounts of water are filtered from the blood into the interstitium where it pools and causes recognizable tissue stretching/swelling.

1.1.2.9. Vascular Volume

Changes to blood volume affect both venous return and correspondingly CO as a reflection of increases in mean circulatory filling pressures. However, when the system is stressed and blood volumes are expanded for a prolonged period of time, the filling pressure and CO will eventually return to normal and relax (Prather, Taylor, & Guyton, 1969). Prather et al. (1969) showed that elevated blood volume for over two hours failed to maintain elevated CO and mean circulatory filling pressures. The veins accommodate increased volume by relaxation of smooth muscle.

1.1.3. Measuring Blood Pressure

Blood pressure measurement is one of the most definitive clinical parameters researchers and medical professionals can use to diagnose and predict CVD morbidity. It is imperative that BP measurements are accurate, as it is a very complex hemodynamic variable that ultimately can have far reaching implications when it comes to the treatment of hypertension. For instance, if a measurement is erroneously low, a patient may be denied drug treatment to prevent future heart attack or stroke, and conversely if the measurement is too high the patient may be placed unnecessarily on lifelong BP lowering treatments (O'Brien, Beevers, & Lip, 2001). The oscillometric method is considered a non-invasive technique for measuring BP. This method allows the examiner to measure systolic and diastolic BP, by observing the oscillations of blood flow, (i.e. pulse) relative to inflation pressures of an external cuff. Other methods of BP measurement include direct or invasive procedures such as using implanted arterial pressure catheters or radiotelemetry, giving the advantage of receiving beat-by-beat waveforms of BP in anesthetized or conscious subjects.

1.1.3.1. Oscillatory Method

In clinical practice, the oscillatory method uses a pressure cuff and gauge, commonly referred to as a sphygmomanometer, placed onto a subject's arm, which is inflated initially to a pressure in excess of the systolic arterial pressure in the brachial artery. For animal experimentation, non-invasive BP sensor technologies include photoplethysmography,

piezoplethysmography and Volume Pressure Recording, which use a variation of the sphygmomanometer which is commonly called an occlusion tail-cuff (Bunag, 1973). The oscillatory method is based on the transmission and recognition of Korotkoff sounds generated by blood flow in the artery (Jorde & Williams, 1986). When the cuff pressure exceeds systolic pressure, blood flow is impeded in the artery in contact with the cuff and no sound is heard. The pressure of the cuff is then slowly released until the cuff pressure and Korotkoff sounds begin to oscillate with the cycle of blood flow in the brachial or tail artery (Kurtz, et al., 2005). The point of maximum oscillation, provides the subject's mean intra-arterial pressure, and systolic and diastolic BP are then estimated from an empirically derived algorithm (Kayrak, et al., 2010). Benefits of using the oscillatory method include the relatively low cost of the equipment; that the subjects can be conscious, surgery or anesthesia are not required, and relatively no chance of injury, infection or death associated with the procedure.

1.1.3.1.1. Errors in Oscillatory Blood Pressure Measurements

Blood pressure measurements provided by manual or semiautomatic instruments, may vary considerably from a subject's 'true' BP due to short-term BP perturbations and incorrect or skewed calibration techniques (Hansen & Staber, 2006). Anxiety and/or stress are a significant source of error when taking BP cuff measurements. White-coat hypertension describes elevated BP measurements seen in up to 25% of subjects examined by health professionals, attributed to the anxiety caused by the exam (Jhalani, et al., 2005). Anxiety is linked to activity in the amygdala, nuclei located in medial temporal lobes of the brain (Rosen & Schulkin, 1998). The amygdala can send direct nerve impulses to the hypothalamus for the activation of the SNS (Karrow, 2006). As discussed previously, the SNS can both directly increase CO and TPR when activated. Therefore it is obvious that anxiety and stress are often associated symptomatically with increased BP. However, this source of error can be lessened by acclimatizing the subject prior to measurement. For rodents, it is suggested that the rodent is placed in a comfortable holder, preferably with a darkened nose to limit the animals' view, and to use a warming blanket and comfortable room temperature to encourage blood flow and overall reduce animal stress (Kurtz, Griffin, Bidani, & Davisson, 2005).

1.1.3.1.2. Intra-Arterial Blood Pressure Catheters

Intra-arterial BP catheters provide real-time BP measurements. This type of measurement is commonly used in intensive-care patient management where BP and blood gas

measurement in real-time is critical to patient survival (Daily, 1995). However, the placement of the catheters can be painful in a conscious subject, and with any invasive technique there is the risk of infection, thrombosis and potential arterial bleeding if a catheter is dislodged. Intra-arterial catheters provide flexibility in experimental investigation, as they can be manipulated in their placement to measure other hemodynamic parameters including atrial, ventricular and venous pressures. A complication of using intra-arterial catheters in experimentation is that anesthesia is required which can depress CV and respiratory function.

1.1.3.2.1. Anesthesia and Intra-Arterial Blood Pressure Measurement

Central systolic BP, measured by aortic catheterization, as opposed to brachial BP measurements, may provide measurement of cardiac afterload and preload and therefore is an important tool in assessing CV pathology and in determining CVD risk (Hope, et al., 2005). In choosing an anesthetic for catheter implantation, researchers choose those with minimal respiratory and CV effects. Volatile anesthetics can change the rhythm, rate and depth of breathing at clinically relevant concentrations (Karanovic, et al., 2009). Heart rate changes in response to periodic changes in lung volume, may be used as a measure of PNS function (Mehlsen, et al., 1987). Isoflurane depresses both the SNS and PNS equally (Kato, et al., 1992). Isoflurane is also known to increase HR and decrease TPR (Eger, 1984). The older volatile anesthetics such as halothane, have little or no effect on vascular tone, whereas isoflurane directly limits coronary vasodilator reserves in coronary resistance vessels (Ciofolo & Reiz, 1999). These older anesthetics have been discontinued for several reasons including their known ability to cause myocardial depression, and hepatotoxicity as seen with halothane (Dale & Brown, 1987). Isoflurane is not metabolized like halothane and does not cause the same pronounced toxic effects. Additionally, myocardial contractility is stable with isoflurane, making it the inhalation anesthetic of choice for many surgical operations (Dale & Brown, 1987).

1.1.4. Hypertension

Hypertension is a medical condition where BP is chronically elevated above set levels of 140 mmHg systolic and 90 mmHg diastolic BP (Carretero & Oparil, 2000). When the word hypertension is used without a qualifier it is defined as the systemic elevation of arterial BP. Hypertension is considered a symptom of CVD and chronic hypertension is a major risk factor for other types of CVD including myocardial infarction (MI) and cerebrovascular accident (stroke) (Bale, 2010).

1.1.4.1. Epidemiology of Hypertension

Kearney et al. reported that hypertension affected approximately 1 billion people worldwide in 2000, likely an underestimation, and that by 2025 this number will increase to nearly 1.56 billion (Kearney, et al., 2005). The prevalence of hypertension in a population is related to several factors reported by Carretero et al.: (1) race, with the highest occurrence found in African American populations (32.4%) followed by Caucasian (23.3%) and Mexican American populations (22.6%); (2) age, since systolic BP rises throughout life in industrialized countries; (3) geographic patterns, for instance, hypertension is most prevalent in the southeastern United States; (4) gender, the development of hypertension is more commonly diagnosed in men, discounting the effect of menopause on this trend; and lastly (5) socioeconomic status, which is inversely related to the prevalence, morbidity, and mortality rates of hypertension (Carretero & Oparil, 2000). Given the global prevalence of hypertension it is disheartening to know that data published recently in the Framingham Heart Study, indicates that even in individuals with normal BP at age of 55, there is still a 90% risk of developing hypertension at some point in their life (Sundström & Vasan, 2005).

1.1.4.2. Pathophysiology of Hypertension

Hypertension is a serious risk factor for CVD. There continues to be uncertainty about the exact pathophysiology of hypertension. There are several known etiologies for hypertension including renal and/or adrenal disease, classified as secondary hypertension. Essential hypertension describes a rise in BP from unknown etiologies. Interrelated factors may contribute, with potential synergism, to increase arterial BP.

1.1.4.3. Risk Factors for the Development of Hypertension

1.1.4.3.1. Essential Hypertension

Essential hypertension remains a major modifiable risk factor for CVD. Its cause has not yet been elucidated and may be due to unknown genetic variations and many non-hereditary factors (Muda, et al., 2005). Healthy lifestyle habits such as maintaining a normal body weight, not smoking, moderate alcohol intake, regular exercise, and healthy diet are individually and jointly associated with a lower lifetime risk of CVD (Djousse, Driver, & Gaziano, 2009).

1.1.4.3.1.1. Race/Ethnicity

It is estimated by segregation analysis that up to 45% of individual differences in BP can be accounted for by ethnicity (Luft, 2002). One cause of hypertension has been linked to a gene responsible for the proteolytic cleavage of angiotensinogen into angiotensin II, one of the most active vasoconstrictive agents (Watt, et al., 1992). African Americans are shown to be the predominant carriers of an AGT gene polymorphism which is directly correlated with an increased risk of developing hypertension (Kumar, et al., 2005). Racial genetic differences have also been identified in ion-transport mechanisms, salt-sensitivities and calcium homeostasis (Douglas, Thibonnier, & Wright, 1996).

1.1.4.3.1.2. Family History and Epidemiology

Hypertension is about twice as common in a person with a hypertensive parent, and epidemiological studies suggest that family genetics account for 30% of a populations' BP (Beavers, 2001). Hyperinsulinemia was significantly associated with a family history of hypertension in healthy children compared to having a family history of other CVD risk factors (Rodríguez-Moran, et al., 2010).

1.1.4.3.1.3. Insulin Resistance (Type I Diabetes)

The prevalence of insulin resistance (IR) in hypertensive individuals is high, and has been shown to be a more predominant risk factor for hypertension in ethnic than Caucasian populations, with and without type 2 diabetes (Huang, et al., 2010). Insulin resistance, resulting in hyperinsulinemia, has been associated with decreased peripheral blood flow and experimental models indicate that IR promotes fibroblastic myocardial growth, increasing left ventricular wall thickness (Lind, et al., 1995). Left ventricular (LV) hypertrophy is a hallmark symptom of prolonged hypertension. Hypertensive patients with LV hypertrophy have been found to have associated IR and impaired insulin-mediated glucose uptake and non-oxidative glucose metabolic capacity (Paolisso, et al., 1995). Impaired insulin-induced endothelial NO production is common during IR, considered to be a non-hereditary risk factors for hypertension as caused by obesity, type 2 diabetes and the metabolic syndrome (Mustafa, 2009).

1.1.4.3.1.4. Smoking

Smoking is considered an important modifiable risk factor for the development of arterial hypertension according to the European Society of Hypertension and the European Society of Cardiology on the management of hypertension (2007). It is estimated that there are currently

3.2 million men and 2.7 million women who smoke in Canada, with rates highest in Quebec and lowest in British Columbia (Lee, et al., 2009). Many studies have well documented an association between chronic obstructive pulmonary disease (COPD) and CVD (Holguin, et al., 2005). The common thread between these two diseases is smoking. The Lung Health Study has shown that age, smoking, gender, marital status, higher diastolic BP, and impaired pulmonary function are strong risk factors for both COPD and correspondingly CVD (Finklestein, Cha, & Scharf, 2009). Long-term exposure to cigarette smoke is shown to impair endothelium-dependent vascular reactivity, induce the proliferation of poorly differentiated smooth muscle cells (SMC) in pulmonary vessels leading to intimal hyperplasia, and reduce the expression of endothelium derived vasodilators such as nitric oxide (NO) (Barbera, et al., 2001). Increased arterial resistance due to SMC proliferation and endothelial dysfunction are hallmarks of hypertension. Smoking is one of the most obvious risk factors in the development of hypertension.

1.1.4.3.1.5. Obesity

The prevalence of hypertension, based on population studies, is about 20% in normal weight individuals and more than 50% in obese individuals (Bo, et al., 2009). In obese subjects, vascular dysfunction, which may include impaired endothelial-dependent vasodilation, decreased arterial compliance and increased inflammatory markers, are detectable prior to the onset of hypertension (Freedman, et al., 2005). Increased epicardial and visceral adipose deposits have been associated with early hypertension, modulating both mineralocorticoid secretion and sympathetic nervous activity through the secretion of adipokines, contributing to increased BP (Natale, et al., 2009). Dyslipidemia can further trigger endothelial dysfunction exacerbating the development of hypertension (Sima, Stancu, & Simionescu, 2009). Obesity is also associated with microalbuminuria. Microalbuminuria is defined as the urinary albumin excretion of 30-300 mg/24 hours, which is considered a risk factor for hypertension, and has also been associated with endothelial dysfunction (Thoenes, et al., 2007). Thoenes et al. (2009) showed that patients with abdominal obesity, had many specific CV risk factors and markers, including elevated BP, blood glucose, and insulin levels. Increases in BP and abdominal obesity have been positively associated with increased body weight and alcohol consumption (Suter, Maire, & Vetter, 1995).

1.1.4.3.1.6. High Alcohol Intake

Many epidemiological studies have demonstrated a linear relationship between alcohol consumption and BP or hypertension, regardless of age, race, gender or the type of alcohol

(MacMahon, 1987). MacMahon (1987) showed that systolic and diastolic BP increased by 3-4 mmHg and 1-2 mmHg respectively per three drinks per day. Alcohol has both vasodilatory and vasoconstrictive actions dependent on plasma concentrations. High alcohol concentration has been shown to augment catecholamine and vasopressin vasoconstriction, and inhibits endothelium-dependent vasodilation (Brizzolara, Morris, & Burnstock, 1994). At low concentrations, alcohol may increase the release of NO and inhibit norepinephrine-induced vasoconstriction (Puddey, et al., 2001). Acetaldehyde, a metabolite of alcohol is also a known vasodilator and is commonly associated with facial flush and hangover headache following ingestion. Alcohol has also been shown to have marked cardiac effects. In hypertensive patients, CO and HR significantly increased after alcohol consumption (Kawano, et al., 1992). The SNS is activated by alcohol, increasing catecholamines and suppressing the baroreceptor reflex (Kawano, 2002) (Abdel-Rahman, Dar, & Wooley, 1985). Alcohol also acts on the endocrine system, increasing the risk for the development of hypertension by increasing plasma renin activity, and stimulating the release of ACTH, plasma cortisol and aldosterone (Howes & Reid, 1986). Urinary excretion is known to increase after consuming alcohol. Detrimentally, increased urinary output of magnesium and calcium are shown in habitual drinkers, possibly contributing to BP elevation and possible arrhythmias (Hsieh, et al., 1992). In conclusion, excessive alcohol consumption has profound and far reaching effects on the CV system.

1.1.4.3.1.7. Excessive Sodium Intake

The renin-angiotensin-aldosterone system is perhaps the most important regulator of sodium, which stimulates its re-absorption in the kidney. Sodium accounts for the majority of the body's extracellular fluid (ECF) osmotic activity. When sodium is at above normal concentrations in the ECF, it brings with it water, thereby increasing total hydrostatic pressures. Since blood plasma is a component of the ECF, increases in hydrostatic pressures will ultimately define BP. Therefore, increased ECF sodium will correspondingly increase BP. Salt-sensitivity will exacerbate sodium as a risk factor for hypertension. The factors that predispose BP salt-sensitivity are chronic kidney disease, obesity, age, African-American ethnicity and diabetes (Cianciaruso, Bellizzi, & Minutolo, 1996) (Anderson, Fedorova, & Morrell, 2008) (Sarafidis & Bakris, 2007). Essential hypertension, as a manifestation of sodium toxicity in susceptible individuals, is prevented by limiting salt intake to less than 2 g per day (Freis, 1976). Salt intake has been shown to lead to left ventricular hypertrophy (LVH) without significantly elevating BP

(Yuan & Leenen, 1991). Yu et al. discovered that increased salt intake elevated BP, potentially due to its other deleterious effects, which included cardiac, vascular, and renal fibrosis and hypertrophy (Yu, et al., 1998). The proposed mechanism for salt-induced tissue fibrosis is increased cytokine expression, such as TGF- β 1 (Yu, et al., 1998).

1.1.4.3.1.8. Sedentary Lifestyles

Many epidemiological and exercise studies give evidence that increased physical activity, of adequate duration, intensity and volume, can lower BP significantly, on its own and in conjunction with pharmacological therapy (Kokkinos, Narayan, & Papademetriou, 2001). Other population studies report that the relative risk for developing hypertension in persons with a sedentary lifestyle, is increased with a 35% to 70% higher BP at rest compared to those whom are physically active (Blair, Goodyear, Gibbons, & Cooper, 1984). The underlying mechanism responsible for exercise regulation of BP is still unknown. Kokkinos et al. reported a 12% reduction in left ventricular mass, after 16 weeks of physical exercise in hypertensive individuals, leading to improved CO (Kokkinos, Narayan, & Colleran, 1995). Reductions in SNS activity, including a decrease in norepinephrine plasma concentrations and therefore reduced TPR also follow exercise (Floras, et al., 1989).

1.1.4.3.1.9. Vitamin D Deficiency

The third National Health and Nutrition Examination Survey (NHANES III) reported, even after adjustments for age, gender, ethnicity and physical activity, that there is a significant inverse association between metabolised serum vitamin D concentration and BP. Vitamin D deficiency impairs calcium and phosphorus homeostasis. Vitamin D is also thought to have an important role in the maintenance of the heart, vascular smooth muscle (VSM), endothelium, stomach, pancreas, brain, skin, gonads and immune system (Holick, 2004). Vitamin D deficiency is associated with obesity and IR (Parikh, et al., 2004), increased vascular resistance and increased carotid intima-media thickness (Duprez, et al., 1993) (Targher, Bertolini, & Padovani, 2006). Potential biological mechanisms which may explain the correlation of vitamin D deficiency to the development of hypertension include vitamin D's ability to suppress renin biosynthesis (Li, et al., 2004) and elevate parathyroid hormone, which increases the risk of arterial calcification (Watson, Abrolat, & Malone, 1997).

1.1.4.3.1.10. Low Birth Weight

Embryonic-fetal development that results in low birth weight (LBW) is associated with reduced nephron endowment, hypertension and renal disease in adulthood (Barker, Osmond, & Law, 1989). Perinatal programming controls nephrogenesis during 34-36 weeks gestational age (Ingelfinger, 2008). Infants born prior to 36 weeks gestational age are at a high risk of LBW. Twin studies reveal that the association between LBW and renal dysfunction is more associated with fetoplacental factors than genetic factors (Gielen, et al., 2005). Moreover, factors that reduce the movement of nutrients via the placenta such as smoking and preeclampsia, additionally increase the risk of LBW (Puddu, et al., 2009). Studies have shown that normotensive humans have on average 1,429,000 nephrons compared to 702,000 in hypertensive individuals (Puddu, et al., 2009). Approximately 10% of very LBW infants develop hypertension after 6 to 12 years (Rodriguez-Soriano, Aguirre, Oliveros, & Vallo, 2005). Lower nephron numbers results in reduced filtration area, glomerular volume and impaired tubular function (Puddu, et al., 2009), indicating impaired renal BP regulation. Other studies report a marked association with LBW and reduced nephron number, with evidence of a higher compensatory glomerular volume (Hughson, et al., 2003).

1.1.4.3.1.11. Elevated Plasma Renin

Renin is the rate-limiting enzyme in the RAS, which as previously discussed, has an established role in regulating blood volume, arterial BP as well as cardiac and vascular function. Renin release ultimately regulates the formation of Ang II, which when acting through its receptor AT₁ increases arterial tone, renal sodium reabsorption, aldosterone secretion and sympathetic neurotransmission (Staessen, Li, & Richart, 2006). RAS over-activation is therefore considered to be a major factor in the pathogenesis of hypertension (Maiba & Feldman, 2003). Other deleterious effects of Ang II activation of AT₁ receptors that increase the risk of developing hypertension include oxidative stress and unregulated cellular growth causing endothelial dysfunction and vascular and ventricular hypertrophy (Brasier, Recino, & Eledrisi, 2002).

1.1.4.3.1.12. Type II Diabetes (Insulin Resistance)

Insulin resistance is a condition where insulin loses its effectiveness at reducing blood sugar, by facilitating glucose uptake and storage in muscle and adipose tissue. Several studies have confirmed that approximately 50-70% of normal weight patients with essential

hypertension have reduced insulin sensitivity (Bao, Srinivasan, & Berenson, 1996) (Ferranini, Buzzigoli, & Bonadonna, 1987)(Srinivasan, et al., 1996). Insulin resistance may lead to the development of hypertension due to altered peripheral resistance, increased SNS activation and increased sodium retention (Scherrer & Sartori, 1997)(Hopkins, et al., 1996). Endothelin-1 (ET-1), a potent vasoconstrictor, is often elevated in obese individuals (Muller-Wieland, et al., 1998). Endothelin elevation compounded with insulin mediated endothelial dysfunction, demonstrated by decreased endothelium-dependent nitric-oxide vasodilatory action, results in increased BP (DeFronzo, 2010). Furthermore, elevated plasma levels of epinephrine and norepinephrine have been observed in obese patients, especially those with arterial hypertension (Weidmann, et al., 1993).

1.1.4.3.1.13. Homocysteine

There is an increasing body of evidence that suggests that hyperhomocysteinemia (hHcy), or elevated plasma concentrations of homocysteine (Hcy), is a modifiable risk factor for the development of essential hypertension. Oxidative stress has been proposed as the primary pathophysiological mechanism through which Hcy contributes to the development of hypertension. Homocysteine increases oxidative stress (Voutilainen, et al., 1999), reduces the bioavailability of NO (Zhang, et al., 2000), stimulates VSM cell proliferation and decreases the ratio of elastin to collagen in the vascular wall (van Guldener & Stehouwer, 2000). All of these properties of hHcy implicate increased vasoconstriction and vascular resistance, as the features of Hcy derived hypertension.

1.1.4.3.2. Secondary Hypertension

Secondary hypertension differs from essential hypertension as it is attributed to an underlying, identifiable cause. Some common causes of secondary hypertension often include diseases, of the renal or endocrine systems, medications or toxicants induced hypertension. Special conditions such as sleep apnea and pregnancy can cause the development of hypertension.

1.1.4.3.2.1. Kidney Disease

Hypertension is a classic symptom of chronic kidney disease (CKD). The loss of kidney function results in a decline in glomerular filtration rate and therefore a reduced ability to regulate body fluids and correspondingly BP. Conditions arising from CKD include: activation of the RAS and SNS, increases in peripheral vascular resistance and BP (Johnson, Alpers, &

Yoshimura, 1992) (Converse, Jacobsen, & Toto, 1992); reactive oxygen species (ROS) inactivation of NO and subsequent endothelial dysfunction (Mallamaci, Tripepi, & Maas, 2004); and sodium overload dependent expansion of extracellular fluid volume causing VSM cell hypertrophy and accelerated damage to kidney tubules and microcirculation (Gu, Anand, & Shek, 1998). Hypertension, in patients with CKD, is almost inevitable, so treatment of the condition aims for a target BP of 130/80 mm Hg, with minimal use of pharmaceuticals such as non-steroidal anti-inflammatories (NSAIDs), cyclooxygenase (COX) inhibitors and amphetamines, known to markedly worsen BP control (Chobanian, Bakris, & Black, 2003).

1.1.4.3.2.2. Adrenal Disease

The adrenal gland is located superior to the kidneys. The cortex of the adrenal gland is responsible for the synthesis of corticosteroid hormones including: aldosterone, cortisol and testosterone. The adrenal medulla is the body's main source for circulating epinephrine and norepinephrine. When diseased, the synthesis of hormones and catecholamines from the adrenal gland is compromised resulting in either an over- or under-abundance of circulating levels. Hyperaldosteronism, is characterized by over-synthesis and secretion of aldosterone, despite high salt and volume retention, which can be predictive of hypertension (Wei, Whaley-Connell, & Habibi, 2009). Cushing's syndrome is another adrenal disease characterized by the over-secretion of cortisol, also associated with the development of hypertension.

1.1.4.3.2.3. Hyperaldosteronism

Elevated levels of aldosterone have been reported in hypertensive patients and hypertensive animal studies (Douma, Petidis, & Doumas, 2008). Elevated aldosterone levels have the following effects leading to the development of hypertension: increased left ventricular mass (Schunkert, Hense, & Muscholl, 1997); endothelial dysfunction indicated by decreasing NO bioavailability, as a consequence of reactive oxygen species generation (Schiffrin, 2006); activated RAS in the brain, increasing sympathetic vascular tone (Yu, Wei, & Zhang, 2008); increased salt appetite and sodium intake through activation of centers in the amygdala further promoting BP elevation (Gomez-Sanchez, 2004).

1.1.4.3.2.4. Cushing's Syndrome

Hypertension is a common symptom of Cushing's syndrome, developed in correlation with increased levels of circulating cortisol. Cortisol activates the RAS, yet renin concentrations are generally normal in individuals with Cushing's syndrome (Suzuki, Handa, & Kondo, 1982).

Activation of RAS by cortisol appears to be mediated by increased AT₁ receptors for Ang II in both peripheral tissues and the brain (Sato, Suzuki, & Murakami, 1994). Evidence also indicates that cortisol increases β -adrenergic receptor sensitivity to catecholamines, increasing vascular tone (Sakaue & Hoffman, 1991). Glucocorticoids, such as cortisol, are also implicated in the elevation of cytosolic Ca²⁺, due to the downregulation of the Na⁺/Ca²⁺ exchanger in VSM cells, causing subsequent vasoconstriction (Smith & Smith, 1994).

1.1.4.3.2.5. Medications

Side effects of medication may also contribute secondary hypertension. Several major classes and types of medications have a risk of increasing BP, in specific risk groups, such as the elderly, and those with pre-existing kidney disease or hypertension. NSAIDs and COX inhibitors block the synthesis of prostaglandins, increase BP in susceptible individuals by about 5 mm Hg (de Leeuw, 1996). The inhibition of prostaglandin synthesis is associated with vasoconstrictor and antinatriuretic effects (Brater, 1999). NSAIDs also inhibit the detoxification and elimination of aldosterone, increasing basal levels and consequently increasing BP (Knights, Mangoni, & Minors, 2006).

Cyclosporine, a calcineurin immunosuppressor, used in organ transplant patients to prevent rejection, causes afferent arteriolar constriction and tubulointerstitial fibrosis (Andoh & Bennett, 1998). Both of these effects increase BP and decrease glomerular filtration rate leading to hypertension. *In vitro*, cyclosporine produces ROS, most notably superoxide and hydrogen peroxide (Lopez-Ongil, Hernandez-Perera, & Navarro-Antolin, 1998). The effects of cyclosporine that are causal to the pathogenesis of hypertension have been effectively eliminated with antioxidant therapy, suggesting that ROS are the mediator of the drug's toxicity (Andoh, et al., 1997).

Erythropoietin (EPO) increases BP in normotensive, healthy subjects as well as CKD patients (Krapf & Hulter, 2009). Erythropoietin dependent increases to BP are considered independent of the hematocrit increase. More recent evidence from animal and human studies suggests that EPO increases platelet cytosolic calcium, required for VSM contraction (Vaziri, Zhou, & Naqvi, 1996) (Tepel, Wischniowski, & Zidek, 1992). Ni et al. (1998) demonstrated that calcium channel blockers attenuate BP increases in EPO treated rats, supporting the theory of Varziri et al. (1996) and Tepel et al. (1992). Moreover, evidence also indicates that EPO may induce hypertension through the release of ET-1 and the vasoconstrictive prostanoid thromboxane

(Bode-Boger, Boger, Kuhn, Radermacher, & Frolich, 1996), and by decreasing endothelial nitric oxide synthase (eNOS) expression in cultured human endothelial cells (Wang & Vaziri, 1999).

Monoamine oxidase inhibitors (MAOIs) are widely used for the treatment of psychiatric disorders including depression, despite long-standing concerns over their hypertensive effects (Rabkin, Quitkin, & McGrath, 1985). MAOIs alone are not considered risk factors for hypertension, but when used in combination with other medications and some types of food, they are likely partially responsible for hypertensive crisis, described as diastolic BP of 120 mmHg (Cook & Katritsis, 1990). Diastolic pressure of this magnitude in humans carries a considerable risk for organ damage. Patients using MAOIs are required to follow a strict dietary regime, including avoidance of foods containing tyramine, and are monitored closely for adverse drug interactions if taking other medications such as sympathomimetics, ephedrine and opioids. Nies (1984) indicated that 15% - 45% of MAOIs, in concert with the intake of tyramine or contraindicated medications, are converted into amphetamines such as serotonin, dopamine and norepinephrine, all known to increase both CO and BP.

1.1.4.3.2.6. Obstructive Sleep Apnea/Hypopnea

Obstructive sleep apnea/hypopnea (OSAH) syndrome is characterized by repeated episodes of apnea (suspension of normal breathing) and hypopnea (very shallow breathing) during sleep (Duran-Cantolla, et al., 2009). Brooks et al. (1997) demonstrated in an *in vivo* canine model that intermittent airway occlusion resulted in small night-time BP increases, gradually leading to sustained daytime hypertension. Duran-Cantolla et al. (2009) summarized that in OSAH patients, a combination of intermittent hypoxemia, RAS and chemoreceptor stimulation as well as sympathetic activation, are potential mechanisms in the pathogenesis of OSAH dependent hypertension.

1.1.4.3.2.7. Preeclampsia

Preeclampsia classically presents itself at approximately 20 weeks gestation with the characteristic features of systolic BP of ≥ 140 mmHg and diastolic BP of ≥ 90 mmHg (ACOG Committee on Obstetric Practice, 2002). The amount of literature available describing the pathogenesis of preeclampsia is overwhelming. However, there are several common features that hallmark the significant physiological differences between pregnant women with normal BPs and those whom develop preeclampsia. The placenta is the focal point to the pathogenesis of the disease. Increased oxidative stress, marked by higher levels of lipid peroxidation,

superoxide (O_2^-) and isoprostanes has been shown to cause placental atherosclerosis (Roberts & Hubel, 2004). Perturbation to the natural killer cell/human leukocyte antigen-C axis causes a marked immune reaction shown to inhibit cell migration into the placenta hindering normal development (Lockwood, et al., 2006). Placental malformation leads to the release of antiangiogenic factors such as soluble fms-like tyrosine kinase-1 and endoglin, both known mediators of endothelial dysfunction (Maynard, et al., 2003) (Venkatesha, et al., 2006). Other culprits identified in the pathogenesis of preeclampsia include decreased levels of vascular endothelial growth factor and placental growth factor hindering normal placental development (Zhou et al., 2002). All of the aforementioned factors contribute to abnormal placentation which further contribute to reduced placental perfusion, vascular dysfunction, capillary damage and potential vasospasms, conditions predictive for the development of hypertension.

1.1.4.3.2.8. Toxicants

Heavy metal exposure is an identifiable risk factor for secondary hypertension. Mercury and cadmium have the most profound effect on the CV system. Mercury is a known promoter of oxidative stress as per its Fenton type reactions with sulfhydryl groups. Mercury induced oxidative stress has been demonstrated to include increased hydrogen peroxide production and glutathione depletion (Lund, et al., 1993). The vascular consequences of mercury exposure and subsequent oxidative stress include: inflammation, thrombosis, VSM cell proliferation and migration, and endothelial dysfunction (Houston, 2007). Even more convincing are epidemiological studies on mercury miners whom have significantly increased systolic BP that is 46% higher than age matched controls, and correlated directly with lipid peroxidation and overall vascular oxidative stress (Kobal, et al., 2004).

Cadmium has been shown in animal studies to cause widespread CV toxicity including aorta and coronary artery atherosclerosis, reductions in CO, cardiac conduction system abnormalities, increased BP and renal tubular dysfunction characterized by proteinuria and chronic renal insufficiencies (Hallenbeck, 1984) (Revis, et al., 1981). The proposed mechanism that leads to cadmium induced hypertension includes increases in catecholamines, sodium retention, increased intracellular calcium and alterations in Na^+/K^+ ATPase (Kristensen, 1989).

1.1.5. Reactive Oxygen Species and Hypertension

Reactive oxygen species are reactive intermediates of molecular oxygen (O_2) metabolism. Most ROS species are chemically reactive because they lack a full complement of

paired electrons in their outer valence shell. Examples of ROS include superoxide radical (O_2^-) and the hydroxyl radical (OH^\cdot). These types of ROS are commonly referred to as oxygen free radicals, because of their free, unpaired valence electron. Unpaired outer valence electrons are always in search of a bonding mate with whom to share electrons thus completing and stabilizing this outer electron shell. Therefore, when ROS come into contact with biological tissues or free molecules they will attack that source of electrons and strip or share electrons with them to ensure electron pairing in their outer valence shell, destabilizing or damaging the substance they contact. Other ROS which have a full complement of paired electrons in their outer shell include hydrogen peroxide (H_2O_2) and hypochlorous acid ($HOCl$). Both H_2O_2 and $HOCl$ are considered reactive, as they can easily convert back into a radical form, as O_2^- or OH^\cdot . Many ROS exist, and they contribute to the development of CVD including hypertension, atherosclerosis, restenosis and diabetic vascular complications (Griendling, et al. 2000) (Touyz & Schiffrin, 2004) (Landmesser & Harrison, 2001). The hypothesis of hypertension by ROS is based upon theories of vascular remodelling and dysfunction which to varying degrees is influenced by ROS (Touyz & Schiffrin, 2004). Intengan and Schiffrin (2001) described that in hypertension, small arteries undergo structural remodelling due to events such as increased cell growth and migration, and include the deposition of extracellular matrix and inflammation.

1.1.5.1. Formation of reactive oxygen species

Oxygen is unique in that it freely accepts electrons generated by physiological oxidative metabolism within cells, and is known to produce ROS including O_2^- , OH^\cdot and H_2O_2 (Auten & Davis, 2009). Other well known ROS that play a role in the pathogenesis of CVD include peroxynitrite ($ONOO^-$), singlet oxygen (1O_2), and peroxy radicals (ROO^\cdot) (Winterbourn & Hampton, 2008). There are many endogenous sources of ROS as well as physiological processes that can result in ROS production including the uncoupling of the electron transport chain in mitochondria, involving normal activity of several enzyme systems such as the cytochrome P_{450} monooxygenase system (Auten & Davis, 2009).

1.1.5.1.1. Superoxide Radical (O_2^-)

Superoxide is a main product of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity and is the primary oxidant that escapes the mitochondrial electron transport chain (Winterbourn & Hampton, 2008). Within the internal mitochondrial membrane, O_2^- may be generated during normal physiological respiration. The reaction involves the reduction of O_2

by the addition of four electrons to produce water. Further chemical reduction of O_2^- can form H_2O_2 and OH^\cdot . Superoxide radical is highly reactive with thiols such as glutathione, homocysteine and sulphates (Winterbourne, 1994), but also with catecholamines and vitamin C (Warren & Ward, 1986). The superoxide radical can be considered an omnipotent ROS, such that on its own it can cause significant oxidant injury to the vasculature. Additionally O_2^- is converted into the extremely toxic OH^\cdot through Haber-Weiss reactions. The O_2^- is a hydrophilic molecule due to its negative charge, and is therefore only able to act locally through internal signalling or paracrine effects.

1.1.5.1.2. Hydroxyl Radical (OH^\cdot)

Produced by the Haber-Weiss or Fenton reactions, O_2^- and H_2O_2 , in the presence of transition metals such as iron, can form the OH^\cdot . The OH^\cdot is considered a highly toxic ROS. It can readily react with membrane lipids causing the formation of even more complex radicals through the process of lipid peroxidation (Auten & Davis, 2009). The OH^\cdot can easily modify deoxyribonucleic acid (DNA), including base and sugar lesions, DNA protein crosslinks as well as strand breaks (Miyamoto, et al., 2007). Those ROS with the ability to modify or damage DNA are likely candidates involved in triggering cellular apoptosis, further contributing to overall cell death. Lipid peroxidation of cell membranes irreversibly damages cells therefore, OH^\cdot is considered a prime culprit in the pathogenesis of CVD such as hypertension and atherosclerosis.

1.1.5.1.3. Peroxynitrite ($ONOO^-$)

In human neutrophils and endothelial cells, superoxide has been shown to react with NO to form $ONOO^-$ (Carrera, et al. 1994) (Kooy & JA, 1994). Additionally, $ONOO^-$ can be protonated to form peroxynitrous acid, another highly reactive and oxidative species, however, having a very short half life, it yields yet again OH^\cdot and nitric dioxide (NO_2^\cdot) (Radi et al. 2000). Peroxynitrite is also a known inducer of lipid peroxidation, indicated by malondialdehyde production and conjugated diene formation (Radi, et al., 1991). Moore et al. (1995) demonstrated that when low-density lipoproteins (LDL) are exposed to $ONOO^-$, lipid peroxidation was promoted, increasing the formation of F_2 -isoprostanes and conversion of the lipoproteins to even more negatively charged moieties. The oxidative hypothesis for atherosclerosis is based upon the oxidation of LDLs, implicating peroxynitrite as a potential therapeutic target.

1.1.5.1.4. Nitric Oxide (NO)

Nitric oxide is considered a key determinant of vascular homeostasis, as it plays a role in vasodilation and endothelium membrane stability (Leopold & Loscalzo, 2009). Nitric oxide can be produced by eNOS in the endothelium, and stimulated macrophages, strongly correlating its production with inflammation, and therefore many forms of CVD such as atherosclerosis (Cohen & Tong, 2010). Recently, endothelial and neuronal NO synthase isoforms expression has been shown in cardiomyocytes (Barouch, et al., 2002). However, Damy et al. (2003 & 2004) showed that changes to the expression of these isoforms are found in CVD conditions such as myocardial infarction and heart failure. In the vasculature, Padmaja and Huie (1993) demonstrated that NO derived reactive nitrogen species react with amino acids, lipid and thiols resulting in various modifications. Sun and Murphy (2010) claim that the nitrosylation of proteins may be cardioprotective, by inhibiting changes to the overall protein structure and function, preventing further, irreversible oxidative/nitrosative modification on thiol groups.

1.1.5.1.5. Singlet Oxygen ($^1\text{O}_2$)

Singlet molecular oxygen is a strong oxidant that is highly reactive towards electron rich organic molecules including proteins, lipids and nucleic acids (Cadet, et al. 2000). Singlet oxygen has been implicated in the toxic effects of ultraviolet (UV) solar radiation exposure, and plays a role in the apoptosis cell signalling cascade (Ryter & Tyrrell, 1998). In biological systems, $^1\text{O}_2$ generation has mainly been attributed to photochemical reactions. Other chemical reactions that may produce $^1\text{O}_2$ are those associated with lipid peroxidation, phagocytosis, and the catalytic mechanisms of peroxidases (Kiryu, et al., 1999).

1.1.5.1.6. Hydrogen Peroxide (H_2O_2)

Hydrogen peroxide is a strong oxidant which is constantly being formed from the mitochondria as well as from enzymatic reactions. Dismutation of O_2^- generates H_2O_2 , which by Fenton reactions in the presence of transition metals, can produce the highly reactive OH^\cdot (Miyamoto, et al., 2007). The formation of H_2O_2 from O_2^- is important biologically because this dismutation provides lipophilicity to the ROS, making it free to cross cell membranes expanding its range of action. Hydrogen peroxide is also known to react with hypochlorous acid (HOCl) to yield $^1\text{O}_2$, (Held, Halko, & Hurst, 1978). The anti-oxidants glutathione peroxidase and catalase are both able to catalyze the conversion of H_2O_2 into water and molecular oxygen, demonstrated by the reaction:



1.1.5.1.7. Peroxyl Radicals (ROO[•])

Peroxyl radicals are formed during a cascade of events that are initiated through lipid peroxidation. When activated, lipoxygenase and cyclooxygenase form lipid hydroxides. As the concentrations of lipid hydroxides rise, the hydroxides are cleaved to produce the ROO[•], known to attack all types of biological molecules including cholesterol, carbohydrates, fatty acids, and proteins (Spiteller, 2006). Peroxyl radical formation has been associated with inflammatory and neurodegenerative diseases such as Alzheimer's Disease and atherosclerosis (Spiteller, 2006)

1.1.5.2. Endogenous Sources of Reactive Oxygen Species

Reactive oxygen species are produced under physiological and pathophysiological conditions. ROS are considered toxic and therefore pathophysiological when the balance between their generation and subsequent detoxification is lost.

1.1.5.2.1. Mitochondria

In the mitochondrial respiratory electron transport chain, a small amount of O₂^{•-} is produced by NADH reductase and cytochrome C reductase (Puddu et al. 2008). Li et al. (1999) described that the rate of ROS generation from the mitochondria is modulated by the potential of the inner mitochondrial membrane, primarily regulated by calcium ion movement. As previously described, excessive production of ROS from the mitochondria causes damage to lipids, proteins and also mitochondrial DNA (mtDNA), which is unprotected and close to the inner membrane, susceptible to ROS insult (Croteau & Bohr, 1997). The synthesis of defective protein subunits of the mitochondrial respiratory chain can result from mtDNA damage, causing a vicious cycle of further oxidative stress and ROS generation (Wei, et al. 1998). Racheck et al. (2006) showed that NO at high concentrations can damage mtDNA by stimulating overproduction of O₂^{•-}, H₂O₂ and ONOO[•].

Oxidized LDL has also been shown to increase mitochondrial O₂^{•-} formation, potentially suggesting its role in the pathogenesis of atherosclerosis (Zmijewski, et al., 2005). Overproduction of ROS from the mitochondria is considered dysfunctional, and has been associated with hypertension as indicated in rat studies (De Cavanagh, et al., 2006).

1.1.5.2.2. Enzymes

A major source of ROS production are physiological enzymes found in inflammatory cells and platelets. Some of the most predominant enzyme sources of ROS in the vasculature include: NADPH oxidase, myeloperoxidase, xanthine oxidase, lipoxygenases, endothelial and inducible nitric oxide synthases (Leopold & Loscalzo, 2009).

1.1.5.2.2.1. NADPH oxidases

NADPH oxidases (Nox) have been identified as major sources of ROS in the vasculature. The Nox family has 7 catalytic homologues, four of which have been identified in the vasculature (Nox 1, Nox 2, Nox 4 and Nox 5), all of which are expressed in endothelial cells, but only Nox 1, Nox 4 and Nox 5 are expressed in VSM cells (Lassegue & Griendling, 2010) (Lassegue & Clempus, 2003). These enzymes produce superoxide via the transfer of electrons from NADPH to molecular oxygen through the following reaction:



The Nox system is activated by many factors including G-protein coupled receptor agonists, cytokines, growth factors, shear stress and ischemia/reperfusion (Frey, Ushio-Fukai, & Malik, 2009). Some of the consequences of Nox derived ROS as described by Frey et al. (2009) include endothelial dysfunction and increased endothelial permeability, inflammation, vascular remodelling, cell growth and migration, apoptosis and senescence, all known contributors in the pathogenesis of hypertension.

1.1.5.2.2.2. Myeloperoxidase (MPO)

Myeloperoxidase, expressed in neutrophils and monocytes, uses chloride as a substrate and H_2O_2 as a co-substrate for the formation of HOCl (Leopold & Loscalzo, 2009). Hypochlorous acid is a potent chlorinating oxidant, and MPO is the only known pathway that synthesizes this reactive chlorinating species in humans (Harrison & Schultz, 1976). Myeloperoxidase catalyzes the oxidation of NO^\cdot to NO_2^- in human plasma (Abu-Soud & Hazen, 2000). Furthermore, MPO activity is in part dependent upon NO^\cdot activity, such that at low concentrations, NO^\cdot reduces MPO into its active form, and at high concentrations, NO^\cdot inhibits MPO by forming a nitrosyl-complex with the enzyme (Abu-Soud & Hazen, 2000). The predominant effects of MPO generated HOCl is that it can oxidize thiols and thiol esters, mediate

the chlorination of amines and unsaturated lipids, and cause the oxidative bleaching of heme groups and iron-sulphur centres (Harrison & Schultz, 1976) (Albrich, McCarthy, & Hurst, 1981).

1.1.5.2.2.3. Xanthine Oxidase

Xanthine oxidase (XO) is expressed only by vascular endothelial cells, and exists in two isoforms, the dehydrogenase and oxidase (Linder, Rapola, & Raivio, 1999) (Stirpe & Della Corte, 1969). Xanthine oxidase produces O_2^- through the catalytic oxidation of hypoxanthine to xanthine and uric acid (Olson, et al., 1974). Only the oxidase isoform generates O_2^- , and its activity is thought to be redox sensitive because under physiological conditions, the dehydrogenase isoform predominates (Nishino, 1997). However, Poss et al. (1996) described that in a hypoxic setting the oxidase isoform will predominate, therefore correlating ischemia with the ability of XO to produce ROS.

Studies have indicated that XO is an important source of ROS that contribute to vascular oxidant stress. For instance, Spiekermann et al. (2003) showed that XO inhibition improved impaired vasodilation and vascular reactivity in patients with known coronary artery disease and hypercholesterolemia. There have also been specific genetic polymorphisms associated with the pathogenesis of CVD, including the A69901C variant of XO, which was identified in a study of 953 hypertensive patients in Japan (Yang, et al., 2008).

1.1.5.2.2.4. Lipoxygenases

Lipoxygenases oxidatively modify biologically active lipids (Leopold & Loscalzo, 2009). The enzyme catalyzes the insertion of molecular oxygen into polyunsaturated fatty acids yielding eicosanoids, including leukotrienes, thromboxanes and through cyclooxygenase, prostaglandin (Samuelsson, et al., 1987). During the transformation of prostaglandin to thromboxane, OH^- and O_2^- are produced. The OH^\bullet is also produced from the lipoxygenase pathway associated with arachidonic acid metabolism (Kontos, Wei, & Povlishock, 1980). Eicosanoids are mediators of inflammatory reactions and have vasoconstrictive and vasodilatory properties, important in the pathogenesis of hypertension and CVD.

1.1.5.2.2.5. Endothelial Nitric Oxide Synthase (eNOS)

Endothelial nitric oxide synthase uncoupling, is clinically relevant in the pathogenesis of hypertension, diabetes mellitus, hypercholesterolemia and atherosclerosis (Pritchard, et al., 1995). Endothelial nitric oxide synthase produces both NO^\bullet and O_2^- through several different pathways. The production of NO^\bullet occurs through electron transfer from NADPH, oxidizing L-

arginine (Leopold & Loscalzo, 2009). When either L-arginine or NADPH are not present in sufficient concentration, eNOS “uncouples” reducing O_2 into O_2^- (Vasquez-Vivar, et al., 1998). Also causing eNOS uncoupling and subsequent production of O_2^- is the release of zinc from a zinc-thiolate moiety of eNOS by $ONOO^-$ (Zou, Shi, & Cohen, 2002).

1.1.5.2.2.6. Inducible Nitric Oxide Synthase (iNOS)

The expression of iNOS is colocalized with oxidized lipids and protein derivatives found in atherosclerotic plaques (Niu, et al., 2001). Within these plaques, iNOS is localized with macrophages and VSM cells (Luoma & Yla-Herttuala, 1999). Just as with eNOS, iNOS uncoupling is thought to be responsible for the generation of ROS. Leopold and Loscalzo (2009) reported that in a diabetic rat model, OH^\cdot and ROO^\cdot were generated by an uncoupled iNOS.

1.1.5.2.3. Other Sources of Reactive Oxygen Species

Reactive oxygen species can originate from various sources throughout the intra- and extracellular spaces. Some small molecules such as thiols, flavins and catecholamines have been known to auto-oxidize and subsequently release oxidative intermediates, around the endoplasmic reticulum, nuclear membrane, and peroxisomes (Muller & Sies, 1987).

1.1.5.3. Reactive Oxygen Species and Vascular Dysfunction

The endothelium regulates vascular homeostasis and function through local mediators that regulate vascular tone, hemostasis and inflammation. There is a mounting body of evidence that indicates that ROS production is directly related to the mechanisms of vascular/endothelial dysfunction. A key feature of vascular dysfunction is vascular remodelling or reorganization of endothelial and/or VSM cells. Furthermore, a dysfunctional vascular system can be diagnosed in relation to the bioavailability and actions of NO. Nitric oxide is a potent vasodilator with a critical role in modulating vascular tone. Nitric oxide is also antithrombotic since it inhibits platelet activity, but also vascular leukocyte adhesion and VSM cell proliferation (Nedeljkovic, Gokce, & Loscalzo, 2003). In summary, the vascular effects of ROS include, impaired vasoreactivity and vascular remodelling.

1.1.5.3.1. Vascular Remodelling

Reactive oxygen species have been shown to regulate to some extent vascular function, through the modulation of cell growth, apoptosis, necrosis, cell migration, inflammation and extracellular matrix protein production (Xu & Touyz, 2006). Rao and Berk (1992) indicated that both VSMC DNA synthesis and cell number increased in response to ROS. VSM cell

proliferation and migration has also been shown to be induced by agonists such as angiotensin II, platelet-derived growth factor and thrombin (Sundaresan, et al., 1995) (Patterson, et al., 1999). In hypertension, Rao and Berk (1992) demonstrated that redox-sensitive growth processes lead to accelerated proliferation and hypertrophy of VSMC, corresponding to further vascular damage and remodelling.

Reactive oxygen species have been shown to modulate vascular structure by increasing extra-cellular matrix protein deposition such as collagen and fibronectin (Touyz & Schiffrin, 2004). Specifically, O_2^- and H_2O_2 have influence over vascular matrix metalloproteinases (MMP)-2 and MMP-9 which cause basement membrane and elastin degradation respectively (Rajagopalan, et al., 1996). Further contributing to vascular remodelling are ROS sensitive inflammatory processes.

Reactive oxygen species have been linked to the expression of proinflammatory molecules such as the monocyte chemotactic protein-1 (MCP-1), interleukin (IL)-1, IL-6, and IL-8, cellular adhesion molecules such as vascular cell adhesion molecule and intercellular adhesion molecule-1, both encoded by endothelial cells and upregulated by cytokines including the interleukins (Muller, et al., 2000). The recruitment of inflammatory molecules in concert with vascular oxidative stress increases endothelial permeability impairing endothelial function and exacerbating vascular damage, as seen in patients with essential hypertension (Kristal, et al., 1998).

Cell death by lipid peroxidation or apoptosis and subsequent cellular reorganization are further hallmarks of vascular remodelling. Increased lipid peroxidation has been identified as a key mechanism for the development of CVD. Subsequent to lipid peroxidation is the formation of highly reactive intermediates, which alter cell function and generate bioactive compounds (Minuz, et al., 2006).

Unsaturated fatty acids such as phospholipids located in cellular membranes, are highly susceptible to peroxidation by ROS. Hydroxyl radicals are able to abstract a hydrogen atom from the fatty acid which then is modified into several reactive substances including aldehydes such as malondialdehyde, and hydrocarbons (Bergamini et al., 2004). These reactive substances are now free to modify other structures. Lipid peroxidation of the plasma and intracellular membranes cause destabilization, resulting in leakage of proteases and Ca^{2+} into the cytosol, further contributing to necrotic cell death (Vanlangenakker, et al., 2008).

Apoptosis is described as programmed cell death including the following identifiable characteristics: membrane blebbing, DNA fragmentation and nuclear condensation. Signals known to be either pro- or anti-apoptotic are regulated by a mitochondrial protein known as Bcl-2 encoded by the B-cell lymphoma 2 gene (Kim, et al., 2006). Dysfunctional mitochondria which can be pro-apoptotic are characterized by their altered transmembrane potential, producing ROS and mitochondrial membrane permeabilization (Gottlieb, et al., 2003). This interaction results in the release of small molecules, the most notable being cytochrome c, activating caspase-dependent apoptotic pathways (Sharpe, et al., 2004). Reactive oxygen species such as H_2O_2 and ONOO^- , in high concentration ($>100 \mu\text{mol/l}$), are pro-apoptotic causing the detachment and shedding of endothelial cells from the extracellular matrix (Li, et al., 1999).

1.1.5.3.2. Impaired Vasoreactivity

Impaired vasoreactivity in hypertension has been linked to decreased NO bioavailability (Touyz & Schiffrin, 2004). Superoxide is known to quench NO because of its ability to transform it into ONOO^- . Peroxynitrite, a very weak vasodilator when compared to NO, has pro-inflammatory properties which makes it pro-thrombotic (Szabo, 2003). Decreased NO bioavailability may be secondary to its impaired synthesis. Reactive oxygen species, as previously discussed, can cause endothelial cell death, leading to reduced expression of eNOS and therefore decreased synthesis of NO.

Vascular tone is also modulated directly by ROS. Somers and Harrison (1999) as well as Yada et al. (2003) described that H_2O_2 acts as an endothelium-derived relaxing factor, with evidence of pulmonary, coronary and mesenteric artery dilation. Yet, spontaneously hypertensive rats have been demonstrated to have enhanced H_2O_2 -mediated contraction of their aortic and mesenteric arteries (Gao & Lee, 2001). Presently it remains unclear what the exact contraction/dilation effects of ROS are with respect to vascular physiological and pathophysiological conditions (Touyz & Schiffrin, 2004).

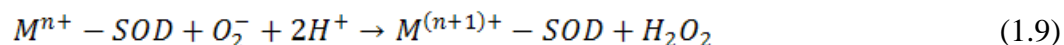
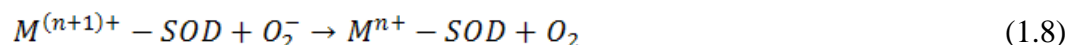
1.1.5.4. Antioxidant Protection from Vascular Dysfunction and Remodelling

Reactive oxygen species are continuously produced during aerobic metabolism. The oxidative stress hypothesis of endothelial dysfunction is measured by the availability of protective antioxidants which prevent and/or limit vascular damage caused by ROS. Antioxidants provide a chemical, detoxification defense to ward off lipid peroxidation, NO inactivation, eNOS uncoupling and apoptosis of endothelial, VSM and myocardial cells.

Antioxidants are classified as either non-enzymatic or enzymatic. The best known enzymatic antioxidant defense systems are superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx). Examples of non-enzymatic antioxidants include vitamin C, vitamin E and carotenoids, polyphenols and alpha-lipoic acid.

1.1.5.4.1. Superoxide Dismutase

Superoxide dismutase exists in three different mammalian isoforms: cytosolic and extracellular copper-zinc SOD (CuZn-SOD and EC-SOD) and manganese SOD (Mn-SOD), localized in the mitochondria (Faraci & Didion, 2004). Each isoform catalyzes the dismutation of O_2^- into H_2O_2 and molecular oxygen as per the following reaction where the metal (M) = Cu (n=1), Mn (n=2), Fe (n=2), and Ni (n=2):



The redox metals contained within the center of the SOD isoform are the source of the catalytic action, reduced by one O_2^- and oxidized back by the second O_2^- (Faraci & Didion, 2004).

Copper-Zinc-SOD is the predominant isoform of the enzyme expressed in high levels in all cells but importantly in blood vessels. In mouse aortas, CU-Zn-SOD accounts for 50% - 80% of total SOD activity (Guo, et al., 2001). Relatively the same expression levels have been reported in human arteries (Guo, et al., 2001). Consequences of Cu-Zn-SOD deficiencies include: increased levels of O_2^- and peroxynitrite, impaired NO-mediated relaxation, increased arterial tone (Didion, et al. 2002).

Manganese-SOD, localized in the mitochondria, a known producer of O_2^- , is the first line of defense against oxidative stress. Landmesser et al. (2001) found that Mn-SOD is found at higher levels in cerebral arteries compared with the aorta or carotid arteries. Packer et al. (2002) reported that mice who do not express Mn-SOD die within weeks after birth.

The only extracellular isoform, EC-SOD, has an affinity for heparan sulfate proteoglycans on cellular surfaces, in the extracellular matrix and basement membranes to which it binds (Marklund, 1984). Heparin treatment can elevate systemic levels of EC-SOD by releasing it from its tissue bound state. EC-SOD is also found between the endothelium and

VSM, with its major source being the muscle since the endothelium has not been shown to express the enzyme (Fukai, et al. 2002). A major function of EC-SOD is to protect NO diffusion from the endothelium to the vascular muscle (Oury, Day, & Crapo, 1996). Interestingly, EC-SOD deficiency has not been correlated with changes to BP, however EC-SOD deficient mice have greater arterial pressures than controls (Jung, et al., 2003). There are some species differences for EC-SOD heparin-binding affinities. For instance, mice and humans have very similar levels of EC-SOD that has a high affinity for heparin in both species (Stralin, et al., 1995). However, in rats, only low levels of EC-SOD have been detected in the vasculature, and what has been expressed does not bind to heparan sulfate under physiological conditions (Faraci & Didion, 2004).

1.1.5.4.2. Catalase

Catalase is an important intracellular H_2O_2 detoxifying antioxidant. Enzymatically, catalase converts H_2O_2 into molecular oxygen and water as per the following reaction:



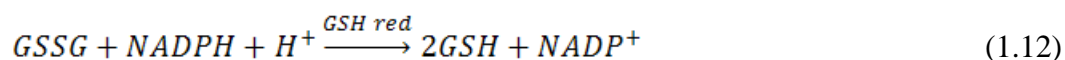
Systematically, in conjunction with SODs, catalase can effectively eliminate the H_2O_2 remaining after O_2^- dismutation. In cases of limited glutathione levels or GPx activity, catalase is very effective at protecting cells from high-level oxidative stress (Wassmann, Wassmann, & Nickenig, 2004).

1.1.5.4.3. Glutathione Peroxidase

Glutathione peroxidase is similar to catalase in its ability to scavenge and reduce H_2O_2 . It can also reduce lipid peroxides to water and lipid alcohols via the oxidation of glutathione (GSH) to glutathione disulfide (GSSG). Thought to be a major defense for low-level oxidative stress, reduced expression of GPx or GSH levels are associated with increased OH^- and lipid peroxy radicals (Wassmann, Wassmann, & Nickenig, 2004). The reaction of the glutathione systems is as follows:



Reduced GSH, the most important regulator of the above reaction, is found in millimolar concentrations intracellularly (Parodi, De Maria, & Roubina, 2007). The oxidized form of GSH, GSSG as per Parodi et al. (2007) represents less than 2% of the total intracellular glutathione pool. The most desirable redox state for glutathione is maintenance of higher levels of GSH relative to GSSG. Changes to the redox state of GSH is compensated by a recycling event initiated by GSH reductase, using NADPH as a substrate converting GSSG back to GSH as per the following reaction:



Consequently the redox state of GSH affects other redox couples such as NADPH/NADP⁺ as well as NADH/NAD⁺ contributing to its known ability to act as a promoter of apoptosis (Parodi, De Maria, & Roubina, 2007).

Glutathione, a tripeptide containing cysteine, glutamate and glycine residues has an affinity, much like any other thiol containing species, to undergo protein modification when exposed to oxidative stress (Kondo, Hirose, & Kageyama, 2009). Their modification includes the formation of protein-disulfides (Pr-S-SG) called S-glutathionylation, a reaction between proteins and GSSG (Kondo, Hirose, & Kageyama, 2009). This modification essentially reduces the availability for conversion of GSSG back to GSH, hindering again the redox state of the antioxidant. Protein modification is reversible via glutaredoxin, reduced by the initial oxidation of GSH, known to play an important role in protecting vascular cells from apoptosis.

1.1.5.4.4. Non-enzymatic Antioxidants

Non-enzymatic antioxidants are rapidly becoming a source of significant pharmaceutical interest for the development of CV drugs which target the oxidative hypotheses, including polyphenols, vitamin C, vitamin E, carotenoids and alpha-lipoic acid. The most important source of the aforementioned antioxidants is dietary.

Vitamin C is water-soluble, found in blood and plasma, reacting as a major antioxidant, in reducing tocopherol radicals and thereby lipid peroxidation in vascular membranes (Fusco, et al., 2007). Vitamin C also has the ability to regenerate vitamin E, a lipophilic moiety residing within lipoproteins and membranes. Vitamin E is believed to significantly protect LDLs against oxidative modifications, contributing to its ability to prevent atherosclerosis (Fusco, et al., 2007).

Carotenoids are also lipophilic antioxidants, whose concentration is inversely related to the levels of isoprostanes, inflammation and atherosclerosis (Block, Dietrich, & Norkus, 2002). The most potent and most studied carotenoid is β -carotene, which is claimed to be able to quench singlet-oxygen radicals (Di Mascio, Murphy, & Sies, 1991).

Polyphenols are available through many dietary sources including fruits and berries, broccoli and cabbage, red wine, chocolate and some teas. In most studies, polyphenols are reported to have low bioavailability due in part to their rapid excretion (Williams, Spencer, & Rice-Evans, 2004). However, their reported antioxidant effects include reduced vascular inflammation and levels of oxidized LDL which maintains interest in their potential application as protectors of CVD (Serafini, et al., 2000).

Alpha-lipoic acid (ALA) is found at highest concentrations, per gram of dry weight in broccoli, spinach and tomatoes, and is accumulated in tissue when consumed (Ghibu, et al., 2009). The antioxidant effects of ALA are vast including its ability to scavenge the OH^\cdot , $^1\text{O}_2$, HOCl and ONOO^- (Ghibu, et al., 2009). Furthermore, ALA is a potent chelator of metal ions such as manganese, copper, iron and zinc (Suh, Moreau, & Heath, 2005).

1.1.6. Homocysteine and Hypertension

1.1.6.1. Homocysteine Metabolism and Reactivity

Homocysteine (Hcy) is a non-protein, sulfhydryl-containing amino acid that is produced from the metabolism of methionine, an essential amino acid. The metabolism of Hcy can be divided into three distinct pathways: (1) the remethylation of Hcy to methionine by the vitamin B_{12} dependent methionine synthase; (2) the transsulfuration pathway, converting Hcy to cystathionine and then cysteine via. vitamin B_6 dependent cystathionine β -synthase (CBS) enzyme; (3) in the liver and kidneys, Hcy can be remethylated back to methionine by betaine-homocysteine methyltransferase (Maron & Loscalzo, 2009). Figure 2 illustrates the Hcy metabolic cycle.

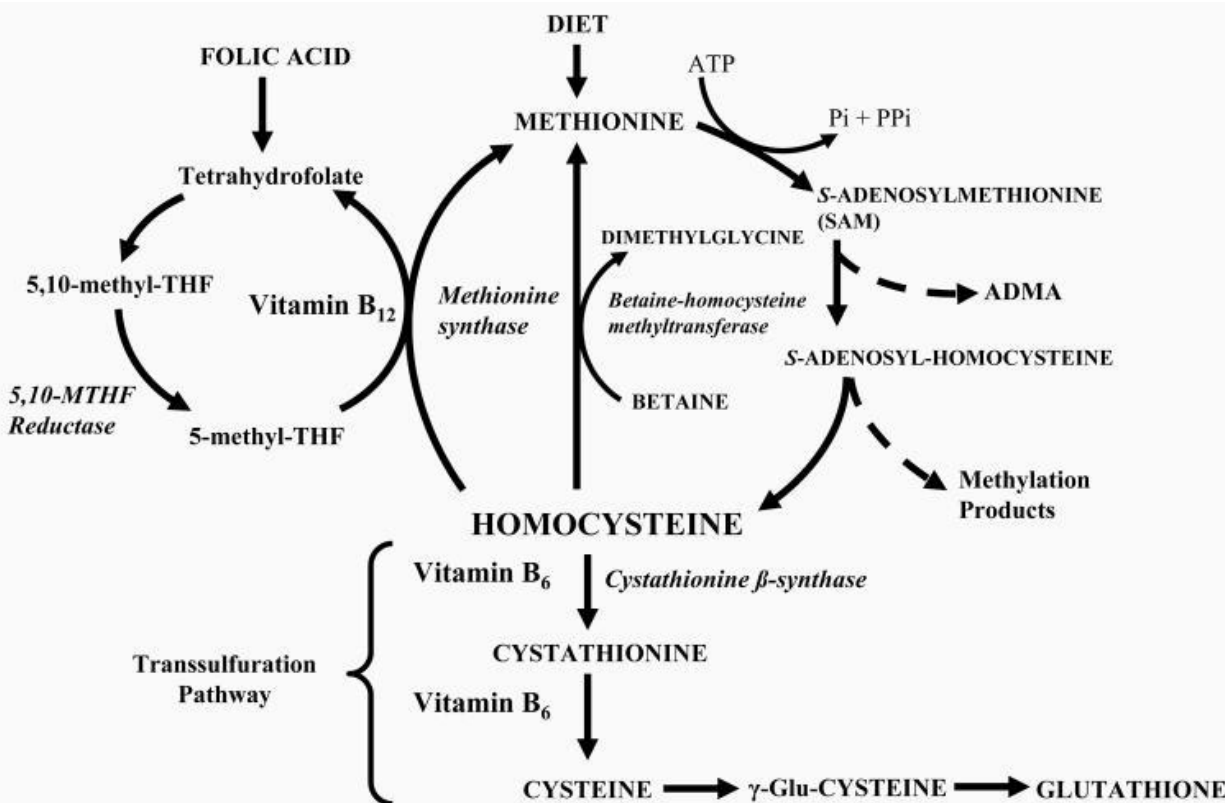


Figure 2 The homocysteine metabolic cycle. (Maron & Loscalzo, 2009), with Permission from Annual Reviews Inc.

Several Hcy species have been identified in human plasma including albumin-(protein)-bound, free circulating disulfides and sulfhydryl forms (Maron & Loscalzo, 2007). However, total Hcy (tHcy) plasma concentrations are commonly reported in the literature as current analytical methodology involves the reduction of Hcy disulfide bonds, quantifying all forms as free tHcy (Maron & Loscalzo, 2009, Perla-Kajan, et al., 2007). The American Heart Association released an advisory statement classifying tHcy plasma concentrations as follows: 5 – 15 $\mu\text{mol/L}$ Hcy as normal, 16-30 $\mu\text{mol/L}$ Hcy as moderate, 31-100 $\mu\text{mol/L}$ Hcy as intermediately elevated and tHcy levels above 100 $\mu\text{mol/L}$ as severely elevated concentrations (Malinow, et al., 1999).

Jacobsen (1998) stated that oxidized Hcy chemical species made up 98-99% of the tHcy in human plasma, where 80-90% of those species are protein bound. In a mouse model of hHcy, Eberhardt et al. (2000) found significant increased ROS accumulation, predominantly O_2^- in aortic tissue as compared with controls. In the literature, it is consistent that *in vivo* and *in vitro*

models treated with Hcy produce elevated amounts of ROS (Eberhardt, et al., 2000, Heydrick, et al., 2004, Postea, et al., 2006, Zhang, et al., 2000).

There are several mechanisms by which Hcy may increase ROS in vascular tissue. Firstly, Hcy is thought to produce ROS when its oxidation rate becomes elevated. Homocysteine contains a free-sulfhydryl group. Such compounds are called “thiols”. Thiols are highly susceptible to oxidation under aerobic conditions where they form disulfides and the ROS hydrogen peroxide according to the general reaction: $2 \text{RSH} + \text{O}_2 \leftrightarrow \text{RSSR} + \text{H}_2\text{O}_2$ (Weiss, 2005). The autooxidation of Hcy through this mechanism is known to form oxidized homocysteine (homocystine), and oxidizes other thiols such as glutathione and cysteine through mixed disulfide formation (Weiss, 2005). However, recent evidence suggests that only a small fraction of oxidized Hcy in plasma results from direct oxidation, so this mechanism is unlikely a major source of Hcy derived ROS (Sengupta, et al., 2001).

Homocysteine has been associated with increased ONOO^- formation through a mechanism which is thought to “uncouple” eNOS (Pritchard, et al., 1995) resulting in endothelial cell membrane lipid peroxidation. Surprisingly, this mechanism has been demonstrated stereospecifically with L-Hcy but not D-isomers (Heydrick, et al., 2004). The suggestion that Hcy causes eNOS uncoupling implies that eNOS is the source for ROS such as O_2^- and ONOO^- .

The vasculature is under oxidative stress when ROS accumulate because of an imbalance between ROS production and protection by anti-oxidants. Homocysteine has been shown to inhibit the activity of cellular antioxidant enzymes including glutathione peroxidase (GPx-1) and SOD (Weiss, 2005). Homocysteine, *in vivo* and *in vitro*, reduces the expression of GPx-1, subsequently preventing its ability to reduce and therefore detoxify H_2O_2 and lipid peroxides into their respective alcohols (Weiss, 2002). Superoxide dismutase is the principle scavenger of O_2^- . It is thought Hcy may interfere with the binding of SOD to the extracellular surface of endothelial cells by causing alterations to endothelial heparan sulfate proteoglycans (Yamamoto, et al., 2000).

Lastly, Hcy has been implicated in neutrophil activation of NADPH oxidases (Weiss, 2005). As previously discussed, the Nox enzymes are a significant source of O_2^- production in the vasculature. Therefore, Hcy activation of Nox enzymes may contribute a significant source of ROS.

1.1.6.2. Hyperhomocysteinemia

Hyperhomocysteinemia, is defined as tHcy concentrations elevated above 15 $\mu\text{mol/L}$. Hyperhomocysteinemia has been strongly associated with the pathogenesis of CVD, and correspondingly has been identified as a contributing factor in four main disease mechanisms including thrombosis, vascular oxidative stress, apoptosis and cellular proliferation (Loscalzo, 1996) (Lubos, et al., 2007, Rounds, et al., 1998, Welch, et al., 1998).

1.1.6.2.1. Etiologies

Genetic, nutritional and medical etiologies have been identified as causing marked elevations in plasma Hcy concentrations. Inherited enzyme mutations, to methylenetetrahydrofolate reductase (MTHFR), essential for Hcy remethylation, as seen in up to 40% of the U.S. population, can increase tHcy by 25% (Qi, et al., 2003). The most common cause of severe hHcy are genetic mutations to the cystathionine β -synthase (CBS) enzyme of the transsulfuration pathway. These mutations are considered inborn errors of metabolism and are seen in 1:200,000 U.S. live births (Maron & Loscalzo, 2007). However, due to the severe elevations of Hcy caused by the CBS mutation, premature death is a common result of associated thromboembolic events (Mudd, et al., 1995).

Three crucial dietary vitamins are required to drive the Hcy metabolic cycle. These vitamins are folic acid, vitamin B₁₂ (cobalamin) and vitamin B₆ (pyridoxal phosphate). Since these vitamins are considered co-factors for Hcy metabolic enzymes, deficiencies in any of these vitamins are associated with hHcy (Brosnan, et al., 2004).

In patients with end-stage renal disease, Robinson et al. (1996) noted that 85% of individuals had elevated Hcy levels as compared to normal controls. Estrogen deficiencies and hypothyroidism have also been associated with hHcy (McCully, 2007) as have psoriasis and lymphoblastic leukemia (Welch & Loscalzo, 1998). Additionally, smoking and certain medications increase Hcy concentrations by depleting folic acid or impairing the synthesis of vitamins B₁₂ and B₆ (Welch & Loscalzo, 1998).

1.1.6.2.2. Oral Methionine Loading

Both experimentally and diagnostically, methionine loading tests evaluate the function of a test subjects' homocysteine metabolism. Hyperhomocysteinemia is diagnosed based on elevated fasting plasma homocysteine levels, 6 hours after oral methionine loading (de Jonge, et al., 2004). Boger et al. (2000) demonstrated that when monkeys were fed a methionine rich diet,

the increase in their tHcy concentrations were associated with impaired NO-dependent carotid artery vasodilation. Wanby et al. (2003) demonstrated that in humans, oral methionine loading was associated with acute elevations in plasma tHcy. In the Wanby et al. (2003) study, patients were given 100 mg of L-methionine per kg of body weight in 200 mL of orange juice in the morning just after their baseline blood test. The patients blood was sampled again 4 hours later (Wanby et al., 2003). Results showed a significant increase in tHcy plasma concentrations (13.7 $\mu\text{mol/L}$ tHcy – baseline: 35.4 $\mu\text{mol/L}$ tHcy – 4 hours after loading; $p < 0.001$) (Wanby et al., 2003).

Elevated fasting plasma tHcy after oral methionine loading indicates a defect in the remethylation metabolic pathway, whereas elevated tHcy levels after loading indicate defective transsulfuration (de Jonge, et al., 2004). To sustain an experimentally relevant hHcy plasma concentration in test subjects that have fully functional methionine metabolic cycles, a high methionine oral load must be sustained over time. De Jonge et al. (2004) state that tHcy reaches its peak concentration 6 hours after methionine loading, which is the normal clinical time to test for hHcy. Fasting plasma tHcy concentrations in healthy subjects, chronically dosed with high methionine, would definitively indicate whether or not overall baseline concentrations had risen.

1.1.6.2.3. Epidemiology

Hyperhomocysteinemia has been established as a risk factor for coronary artery disease (CAD), MI, stroke, venous thromboembolism and peripheral vascular disease through numerous epidemiological reports (Maron & Loscalzo, 2009). A meta-analysis of 27 studies showed that there was an increased, incremental risk of CAD with every 5 $\mu\text{mol/L}$ increase in tHcy concentrations (Boushey, et al., 1995). Boushey et al. (1995) claimed that, hHcy was attributable for 10% of CAD risk in the population, and therefore 50,000 deaths from CAD could be eliminated by Hcy reduction. Supporting these findings was the Homocysteine Studies' Collaboration meta-analysis, which reported a 19% reduction in the risk for stroke and 11% reduction in the risk for ischemic heart disease per 3 $\mu\text{mol/L}$ tHcy reduction (Homocysteine Studies Collaboration, 2002).

1.1.6.3. Homocysteine and Vascular Dysfunction

Vascular dysfunction as it relates to the pathogenesis of hypertension is predominantly characterized by the health and well being of the vascular endothelium. Endothelial dysfunction is predictive of CV pathologies, and is most commonly diagnosed when there is evidence of

impaired vasodilation (Cai & Harrison, 2000). However, since BP is a product of TPR, other factors such as decreased lumen size due to atherosclerotic lesions or thrombosis, are other mechanisms by which Hcy may contribute to the pathogenesis of hypertension.

1.1.6.3.1. Impaired Vasoreactivity

Humans with hHcy have impaired endothelium-dependent vasodilation (Tawakol, et al., 1997) (Chambers, et al., 1999). Reactive oxygen species production in hHcy, quench NO bioavailability both *in vivo* and *in vitro* (Weiss, 2005). The highly reactive O_2^- , produced intracellularly, is in a prime location to react with NO, also produced within the endothelial cell, to form $ONOO^-$. In cultured endothelial cells, Hcy alters vasoreactivity by decreasing the production and/or bioavailability of mediators such as ET-1, NO, and prostacyclin, a vasodilatory eicosanoid (Demuth, et al., 1999, Upchurch, et al., 1997, Wang & Vaziri, 1999).

Another mechanism for hHcy inhibition of NO production is caused by asymmetric dimethylarginine (ADMA) (Austin, et al., 2004). Asymmetric dimethylarginine is a bi-product of methionine metabolism (Figure 2), caused by increased S-adenosylmethionine-dependent methylation of protein residues, or by decreased hydrolyzation and subsequent renal excretion of ADMA (Vallance, 2001). Plasma levels of ADMA rise rapidly after acute methionine loading in humans, correlated directly with impaired vasorelaxation (Boger, et al., 2000). ADMA is a known eNOS inhibitor, and also promotes the uncoupling of eNOS, directly increasing vascular oxidative stress, again reducing the bioavailability of NO (Boger, 2003). In hHcy monkeys, elevated plasma ADMA was associated with impaired relaxation of the carotid arteries (Boger, et al., 2000). Also, in rat models and human subjects given oral methionine, elevated plasma ADMA was correlated with impaired endothelial function (Boger, et al. 2001) (Stuhlinger, et al., 2003). Elevated plasma ADMA is an emerging risk factor for hypertension, hypercholesterolemia, hHcy, diabetes mellitus and CVD (Boger, 2003).

The expression of MCP-1 and IL-8 in human aortic endothelial cells, is also induced by Hcy (Podder, et al., 2001). These inflammatory mediators significantly alter the vasoreactivity of pulmonary arteries (Podder, et al., 2001). Zhang et al. (2007) showed that Hcy, in a dose-dependent manner, decreased cyclic guanosine monophosphate levels, a second messenger normally increased by ACh to facilitate vasodilation, thereby inhibiting the endothelium's vasodilatory response to ACh.

1.1.6.3.2. Thrombosis

Thrombosis, as induced by Hcy, is due to its procoagulant activity and its known ability to alter anticoagulant mechanisms (Lee & Prasad, 2002). Thrombosis and subsequent occlusion of the carotid artery in hHcy mice happened 50% faster than in control mice (Wilson, et al., 2004). The endothelium plays a role in vascular homeostasis by maintaining balance between pro- and anti-thrombotic pathways (Thrambyrajah & Townsend, 2000). Rogers (1998) described that the endothelium uses the following mechanisms to create an antithrombogenic surface: (1) production of tissue plasminogen activators (t-PA), which limits plug formation and fibrin deposition (2) inhibition of platelet activation by NO, prostacyclin and enzymes which cleave ATP; (3) regulation of heparan sulfate, thrombomodulin and protein C activity (Rogers, 1988). Homocysteine has been shown to disrupt all of these mechanisms, predisposing hHcy patients to thrombosis.

Firstly, in cultured endothelial cells, Hcy forms disulfide bonds with a cysteine residue on the endothelial cell surface receptor for t-PA, blocking its binding, leading to decreased plasmin generation, promoting fibrin deposition and ultimately thrombosis (Hajjar, et al., 1998). Secondly, Hcy alters vasoreactivity and promotes thrombogenesis by decreasing the production and/or bioavailability of mediators such as NO and prostacyclin. Lastly, aortic thrombomodulin activity was decreased in hHcy monkeys fed a high-methionine diet, reducing the ability to activate protein C which limits its anti-thrombotic activity, anti-apoptotic and antiinflammatory activities (Lentz, et al., 1996).

1.1.7. Heparin

For many years heparin has been widely used in the treatment and prevention of both arterial and venous thrombosis. Only recently has the focus shifted onto different forms of heparins, including low-molecular weight heparins (LMWH) and the use of different means of systemic delivery such as oral heparins.

1.1.7.1. Chemistry

Heparin and heparan sulfate belong to a family of glycosaminoglycans (HS-GAG) biosynthesized through the attachment of monosaccharides to a main core protein in the Golgi (Sasisekharan & Venkataraman, 2000). Heparin is a highly sulfated polysaccharide commonly isolated from mast cells, while heparan sulfate is a less sulfated polysaccharide found on cellular surfaces, including endothelial cells and on the extracellular matrix (Sasisekharan &

Venkataraman, 2000). HS-GAGs are characterized biochemically by a linear chain of anionic repeating units of uronic acid and glucosamine residues (Skidmore, Guimond, Rudd, Fernig, Turnbull, & Yates, 2008). Heparin has a molecular weight of approximately 60-100 kDa, 750-1000 kDa if the core protein is included (Li & Vlodavsky, 2009). However, the heparin produced from mast cells undergoes degradation prior to being a completed molecule, as Li and Vlodavsky (2009) report with its final molecular weight of 5-30 kDa. Low-molecular weight heparins are prepared commercially through further degradation or fractionation of the main heparin GAG chain with molecular weights less than 8 kDa (REF).

1.1.7.2. Activity

The main studied activity of HS-GAGs are as potent anticoagulants due to their polysaccharide sequence which specifically binds to antithrombin, inhibiting a variety of coagulation factors including factor Xa, and heparin cofactor II, a thrombin inhibitor. Due to the high charge of HS-GAGs, they readily bind to proteins and the vascular wall. Interactions between HS-GAGs and other vascular structures including enhanced protein-protein interactions, sequestering of protein ligands on endothelial cell surfaces and in the extracellular matrix, act as co-receptors for growth factors (Whitelock & Iozzo, 2005). Furthermore, HS-GAGs protect proteins from degradation (Hashimoto, et al., 1997), mediates protein internalization (Gingis-Velitski et al., 2004) and regulate protein membrane transport (Dowd, Clooney, & Nugent, 1999).

In addition to its anticoagulant properties, HS-GAGs have been shown to release EC-SOD and inhibit platelet endoperoxide metabolite formation thereby reducing superoxide concentrations (Karlsson & Marklund, 1987), decrease the accumulation of vascular calcium (Vasdev, Prabhakaran, & Sampson, 1991), reduce ROS generation as related to the activation of prostaglandin and phospholipase biosynthesis. Using xanthine and xanthine oxidase as ROS producers to cause *in vitro* endothelial cell damage, Hiebert and Liu (1990) demonstrated that heparin significantly increased cell viability and decreased lactate dehydrogenase release, thus reducing cellular oxidative injury. Heparin treatment has also repeatedly been shown to lower BP, when administered daily to spontaneously hypertensive rats, characterized by a significant decrease in TPR and increases in CO (Mandal, et al., 1978). Heparin's antihypertensive effect, excluding any effect on hematocrit, include increased endothelial-derived nitric-oxide

vasodilation and inhibition of ET-1 and consequently VSM cell contractility (Mandal, Lyden, & Saklayen, 1995).

1.1.7.3. Administration

Primarily, heparins are administered by intravenous or subcutaneous routes. Experimentally, oral heparin administration has also been shown as a viable alternative. Jaques et al. (1991) and Hiebert et al. (1993) using a rat model, supported findings of Engelberg (1995) that oral heparin is absorbed. Furthermore, the endothelium has been shown to be the target organ for orally administered heparin. Hiebert et al. (1993) showed heparin, within 6 minutes of intragastric administration was detected in association with rat endothelium, and that oral heparins have antithrombotic activity in rats. These results suggest that plasma concentrations of orally administered heparin would be dependent on its uptake and release by the endothelium. Interestingly, oral heparins have also been seen to have effects such as lowering BP in spontaneously hypertensive rats (Vasdev, et al., 1992).

1.2. **RATIONALE AND HYPOTHESIS**

Essential hypertension is an important modifiable risk factor for CVD whose specific causes are difficult to fully elucidate. The difficulty arises due to the multi-factorial nature of BP control. Firstly, BP is a product of both CO and TPR. Therefore, changes to the reactivity of the vasculature and cardiac function can both significantly affect BP. Since the most significant and immediate changes to BP arise through the vasoactivity of arterioles, which are considered the primary resistance vessels in the vasculature, studies about hypertension tend to focus on their relationship to TPR. However, increases in BP due to changes in TPR can in concert cause adverse changes in cardiac structure and function. For instance, increased arterial stiffness can lead to left ventricular dysfunction, such that subjects would show a marked increase in left-ventricular end-diastolic pressure ultimately leading to ventricular hypertrophy and cardiac failure. Therefore, full hemodynamic investigations are ultimately required to elucidate how and by what timeline essential hypertensive mechanisms affect BP.

There has been an increasing body of evidence implicating hHcy as having a causal relationship to the development of hypertension (Rodrigo, et al., 2003) (Tyagi, et al., 2005) (Davis, et al., 2001) (Sutton-Tyrrell, et al., 1997). The exact mechanism by which hHcy is causal to hypertension is unknown but in numerous studies it has been shown to limit the

bioavailability of NO (Boger, et al., 2001), increase smooth muscle cell proliferation and impair vascular elasticity by reducing elastin/collagen ratios (Stehouwer & van Guldener, 2003) and furthermore is deleterious to endothelial cells causing endothelial dysfunction (Zhang, et al., 2000). Each of these proposed mechanisms correspond to increases in TPR and the development of hypertension.

Hyperhomocysteinemia has also been shown to cause adverse cardiac remodelling and heart failure in association with hypertension (Joseph, et al., 2003) (Sundstrom, et al., 2004) (Bokhari, et al., 2005) (Devi, et al., 2006). Joseph et al. (2003) claim that hHcy induces left ventricular hypertrophy, myocardial fibrosis and stiffness. The consequences of cardiac remodelling are impaired systolic and diastolic function, such as low LV ejection volumes (Cesari, et al., 2004). However, there is limited hemodynamic evidence available that comprehensively describes the consequences of hHcy cardiac remodelling.

All of the aforementioned CV effects of hHcy are based on an oxidative hypothesis implicating Hcy thiol auto-oxidation, eNOS uncoupling, activation of Nox enzymes and antioxidant inhibition of GPx-1 and SOD as being contributing factors to its association with increased levels of ROS. Voutilainen et al. (1999) clearly demonstrated that hHcy is positively associated with increased oxidative stress, supported by evidence of increased F₂-isoprostane levels, a specific marker of lipid peroxidation. Furthermore, vitamic C administration, used as an anti-oxidant prevents Hcy dependent endothelial dysfunction in terms of NO inactivation, supporting the view that increased oxidative stress is integral in the pathogenic mechanism of hHcy (Kanani, et al., 1999).

The oxidative hypothesis describing Hcy's causal relationship to hypertension is the key factor that is essential for targeting potential therapeutics. Heparin has been increasingly described as having antioxidant activity, which affords protection to the endothelium from oxidative stress. It has been suggested that heparin acts as an antioxidant by facilitating the release of SOD from the endothelium surface to act in the plasma as an ROS scavenger (Karlsson & Marklund, 1987), and that heparin decreases normal ROS generation by inhibiting prostaglandin and phospholipase biosynthesis (Vasdev, Prabhakaran, & Sampson, 1991). Hiebert and Liu (1990 and 1994) demonstrated specifically that heparin or dextran sulfate, a compound very similar to heparin, protects endothelial cells *in vitro* from ROS damage generated by

xanthine and xanthine oxidase. However, there is no published data which shows that heparin affords antioxidant like protection to endothelial cells from Hcy generated ROS production.

Based on the above background knowledge it is hypothesized that elevated levels of plasma homocysteine will cause oxidative stress leading to endothelial cell dysfunction, resulting in the development of hypertension and that heparin administration will attenuate these effects.

All of the potential pathological mechanisms by which Hcy may have a causal relationship to hypertension are correlated to it being an inducer of oxidative stress in the CV system. The controversy over the specific mechanism which contributes to Hcy induced hypertension is confounded by a lack of hemodynamic information that can both structurally and chronologically differentiate between pathological events. Therefore, a full hemodynamic evaluation of an animal model of hHcy could provide a comprehensive overview of vascular and cardiac function in relation to total plasma levels of Hcy. Furthermore, evidence of CV oxidative stress should be associated with hemodynamic changes. Total oxidative stress can be indirectly quantitated by measuring serum malondialdehyde (MDA), a product of lipid peroxidation related to lipid membrane damage. As oxidative stress is postulated to correspond directly with Hcy mediated increases in BP, MDA measurements taken in systems (1) which provide BP control, (2) organs which produce a significant amount of antioxidant, and (3) large blood vessels which receive a significant volume of blood under high pressure would provide a holistic evaluation of important body systems which has not been studied previously. Further evaluation of GSH concentrations in select tissues and plasma could provide an indication of possible thiol oxidation and mixed disulfide formation between GSH and Hcy, or its depletion via the detoxification of ROS. While MDA suggests oxidative stress via lipid peroxidation, ROS are also known to promote cell death by apoptosis. Since endothelial cells are integral in BP control, evaluation of their apoptosis would provide further evidence of vascular dysfunction. Lastly, because heparin has been suggested to have antioxidant activity, its delivery into an *in vivo* model of hHcy should attenuate CV oxidative stress and any correlated hemodynamic changes.

1.3. OBJECTIVES

Therefore the specific objectives of this study are to:

1. Develop an *in vivo* animal model of hHcy by providing a high-methionine diet, demonstrated by measuring fasting total plasma Hcy concentrations.
2. Evaluate the correlation between increasing plasma levels of Hcy to the hemodynamics of anesthetized animals.
3. Determine if Hcy has a causal role in the development of hypertension and if heparin attenuates its effects.
4. Determine whether Hcy induces oxidative stress by measuring serum and tissue MDA and plasma and tissue GSH concentrations and whether heparin attenuates its effects.
5. Determine whether Hcy induces endothelial cell apoptosis and whether heparin attenuates its effects.

2.0. MATERIALS AND METHODS

2.1. MATERIALS

L-cysteine, DL-homocysteine, L-glutathione reduced, L-methionine, D-penicillamine, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), dithiothreitol (DTT), propidium iodide (PI), hoescht 33258, tetra ethoxy propane (TEP), sodium dodecyl sulfate (SDS), glacial acetic acid (HAc), triton-X-100 (Tx), sulfosalicylic acid, and ethylene diamine tetra acetic acid (EDTA) were obtained from Sigma Chemical Company, St. Louis, MO, USA. Bio-Rad protein assay was acquired from Bio-Rad, Hercules, CA, USA. DeadEnd™ Fluorometric TUNEL System was obtained from Promega, Madison, WI, USA. Thiobarbituric acid (TBA) was purchased through Helixx Technologies, Scarborough, ON, Canada. Halocarbon Products Corporation, River Edge, NJ, USA provided Halothane and Baxter Corporation, Toronto, ON, Canada provided AErrane (isoflurane, USP) for anesthetic use. All other chemicals are of reagent grade and were obtained from VWR International Inc., West Chester, PA, USA. Homocysteine reagent pack for use with the Abbott IMx Instrument for the detection of total homocysteine in plasma was obtained from Abbott Laboratories, Abbott Park, IL, USA.

2.2. METHODS

2.2.1. Study Design

2.2.1.1. High Methionine Diet Induced Changes in Fasting Plasma Homocysteine Levels and Hemodynamic Parameters

This study was designed to investigate the effect of a high methionine diet on fasting plasma Hcy levels and hemodynamic parameters in male Wistar Kyoto (WKY) rats. The study included 4 diet treatment groups at 2, 4, 6 and 8 weeks exposure, as well as 2 age-matched groups at 2 and 8 weeks fed a control rat diet (n = 5 – 6 per group).

2.2.1.2. Hemodynamic Responses to Different Inhalation Anesthetics

This study was designed to investigate if the type of inhalation anesthetic used during surgery produced any differences in the hemodynamic parameters of male Sprague Dawley (SD) rats fed a high methionine diet for 2 or 4-weeks, including 2 week age-matched controls (n = 5 – 6 per group).

2.2.1.3. Effect of Oral Heparin on Homocysteine Induced Changes to Hemodynamic Parameters, Lipid Peroxidation, Reduced Glutathione Concentrations and Endothelial Cell Apoptosis

This study was designed to evaluate the effect of oral heparin treatment (1 mg/kg/48 hours) on the hemodynamics, lipid peroxidation status, reduced glutathione (GSH) concentrations and endothelial cell apoptosis in male WKY rats with elevated fasting plasma homocysteine levels (n=5 per group).

2.2.1.4. Definition of Variables

The following variables, used throughout this work and are defined as follows:

- Hyperhomocysteinemia (hHcy) – significantly elevated fasting plasma Hcy levels compared to control.
- Hypertension – significantly elevated arterial blood pressure compared to control.
- Oxidative stress – significantly lowered GSH concentrations and/or increased malondialdehyde levels.

2.2.2. Diet Model of Hyperhomocysteinemia

Male WKY rats were fed a modified diet to induce hHcy. Rats were fed a diet of normal rat chow ad libitum, which already contains an essential 0.33% methionine to which an additional 1.7% w/w L-methionine was added for a final concentration of 2.03% methionine. The rats were separated into experimental groups of 2, 4, and 8 weeks exposure. Control rats were age matched to the 2 and 8 week exposure group. Body mass was recorded for each experimental animal prior to surgery.

2.2.3. Hemodynamic Investigation

2.2.3.1. Surgery and anesthesia

At the end of each diet or control period the rats were anesthetized with isoflurane or halothane at 5.0% / L O₂(g) to induce, and 2.0% / L O₂(g) for maintenance of surgical anesthesia. To perform hemodynamic studies, a Millar MikroTip Pressure Catheter was positioned directly at the site of pressure measurement in either the LV or outside of the aortic arch in the right common carotid artery. The MikroTip catheter was appropriate for insertion into the rat carotid artery as the transducer size was 2 French and the wire thread through the vasculature measures at 1.5 French. The pressure catheter was inserted through the right common carotid artery and

advanced into the LV. The pressure catheter was allowed to normalize for 30 minutes in the LV at which point it was retracted into the carotid artery where it was stabilized for 15 minutes until the end of the hemodynamic measurement.

Once the catheter was removed from the animal, arterial blood was collected from a cannula placed into the left common carotid artery. A total of 7 ml of blood was drawn into vacutainers appropriate for future assay. For assays requiring plasma, 5 ml of blood was collected on ice in EDTA tubes. These tubes were divided into 3 ml of plasma for HPLC analysis and 2 ml for Hcy determinations. For the MDA assay, the remaining 2 ml of arterial blood was collected in a serum collection tube. Following bleeding, the animal was sacrificed and the mesentery, first order mesenteric artery branching directly off of the aorta, the aorta, renal artery, kidney and heart were rapidly excised, gently cleaned of blood and debris and stored appropriately based on the assay they were collected for.

2.2.3.2. Data collection

Data was acquired on a Biopac Data Acquisition system and all hemodynamic measurements were made assessing the data on AcqKnowledge software. Left ventricular (LV) hemodynamics were assessed during the last 5 minute segment of the 30 minute normalization period. Left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP), left ventricular developed pressure (LVDP) and the myocardial index of ventricular contraction (+dp/dt) and relaxation (-dp/dt) were assessed. Both the rate of contraction and relaxation were assessed as the maximal slope of the LV pressure waveform either during the rising/contraction or falling/relaxation phase. The LVSP was determined as the maximum pressure reached during ventricular ejection and the LVEDP was assessed as the pressure immediately following atrial contraction and ventricular filling. The LVDP was the difference between the maximum and minimum pressures recorded from the LV.

Arterial pressures were recorded and analyzed with AcqKnowledge software. Arterial pressure was recorded in the last 5 minute recording segment during the 15 minute stabilization period when the pressure tip was in the common carotid artery. Diastolic pressure (DP) was seen as the minimum pressure of the arterial waveform. Systolic pressure (SP) was recorded as the maximum pressure of the arterial waveform. Pulse pressure (PP) was calculated as the difference between systolic and diastolic pressures, and mean arterial pressure (MAP) was calculated as $DP + 1/3 PP$.

2.2.4. Oral Heparin Administration

Unfractionated bovine lung heparin was dissolved in water at a concentration of 5 mg/ml. The dosage of oral heparin was 1 mg/kg/48hrs for 8 weeks. Heparin was given by oral gavage using a steel needle with a blunted end, 7.5 cm in length, to which a 1 ml syringe was attached. Control animals were given 0.4 ml saline by oral gavage every 48 hours.

2.2.5. Detection of Apoptotic Endothelial Cells

2.2.5.1. Tissue collection, preservation and storage

Endothelial cells were assessed for apoptosis in the first order mesentery artery, renal artery and the aortic arch. After the tissues were removed they were fixed for 24 hours in 10% buffered formalin, then cryoprotected by dehydrating the tissue in 30% sucrose solution for a minimum of 72 hours. The tissue was then embedded in optimal cutting temperature (OCT) medium and fast frozen in liquid nitrogen. Frozen samples were kept at -20°C until they were cut by a cryostat and mounted on permafrost slides (VWR). Slides with tissues were kept at -20°C until stained.

2.2.5.2. TUNEL staining protocol

TUNEL staining procedure was carried out following the manufacturer's specifications from Promega's DeadEnd™ Fluorometric TUNEL system. In brief, slides with tissues were washed twice with phosphate buffered saline (PBS) at pH 7.4, after which the tissues were incubated for 8-10 minutes in 20 µg/ml proteinase K solution. After an additional wash with PBS, tissues were fixed in 4% paraformaldehyde. To detect apoptosis tissue sections were exposed to 25 µl of equilibration buffer for 5-10 minutes. Positive controls were prepared by fragmenting chromosomal DNA with DNase 1 for 10 minutes, which exposes multiple 3'-OH DNA ends for labeling by the TUNEL method. Fluorescence labeling of apoptotic EC was accomplished by incubating all tissue sections, except the negative controls, with rTDT incubation buffer for 1 hour in a humidified chamber. Negative controls were treated in the same fashion except the incubation buffer used for treatment was void of the rTDT enzyme. Immersing the tissue sections in an inorganic sodium chloride/sodium citrate solution for 15 minutes terminated the reaction. After washing several times in PBS, the propidium iodide (PI) nuclei stain was added to tissue sections and incubated at room temperature in the dark for 15 minutes.

Samples were analyzed using a fluorescence microscope with a standard fluorescein filter set to view the TUNEL green fluorescence at 520 ± 20 nm and the red fluorescence of the PI nuclei stain at >620 nm. The nuclear stain fluoresces red, and the TUNEL stain fluoresced green, therefore when overlaid cells are seen to fluoresce yellow, and were counted as apoptotic ECs. Cells that failed to fluoresce yellow when images were overlaid, but still displayed the red nuclear stain were counted as non-apoptotic ECs. Slides were mounted with antifade and sealed with coverslips for storage at 4°C until images were taken. All yellow cells, identified on the lumen side of the basal lamina were considered positive for apoptotic endothelial cells. Positive stained apoptotic endothelial cells were counted and expressed as % apoptotic EC/tissue/animal group.

2.2.6. High Performance Liquid Chromatography Assay of Sulfhydryl Amino Acids

Ellman's reagent, 5-5'-dithiobis(2-nitrobenzoic acid) (DTNB) is prevalently used for the detection of thiol groups as it reacts with these groups to give a mixed disulfide which can be quantified by its UV absorbance (1). Combining the effects of DTNB with precise UV detection by high performance liquid chromatography (HPLC), permits for the chromatographic separation of the derivatized thiols, thus allowing for quantitative, sensitive and precise measurements of free reduced sulfhydryl concentrations in biological matrices. Dithiothreitol (DTT) was used via HPLC chromatographic separation in the same fashion as DTNB.

2.2.6.1. Instrumentation

Chromatographic analysis was performed with a Hitachi D-7000 HPLC system which was composed of a L7400 UV detector, L7200 auto-sampler, L7100 pump and water bath. Data was collected digitally with the Hitachi model D7000 HSM chromatography data station software.

2.2.6.2. Preparation of reagents

Mobile phase A was prepared from a stock solution of 0.5 M KH_2PO_4 adjusted to pH 3.89 with orthophosphoric acid, double distilled water (ddH_2O) and HPLC grade methanol. The final composition of the mobile phase was 12.5% methanol (v/v), 100 mM KH_2PO_4 at a final pH of 3.89. The mobile phase was filtered with a nylon membrane filter with a pore size of 0.45. Mobile phase B was used to elute excess DTNB and DTT from the column comprised of 40% HPLC grade methanol (v/v), 100 mM KH_2PO_4 , pH 3.89 and filtered as described above. Mobile phases were stored at 4°C between uses.

A 10 mM stock solution of DTNB was prepared by dissolving 396 mg of DTNB in 100 ml of 0.5 M KH_2PO_4 , pH 7.2, stored at 4°C and used within a month. A 10 mM stock solution of DTT was prepared by dissolving 15.4 mg of DTT in 10 ml of distilled water, which was refrigerated and used within 2 weeks. A solution of 5% (w/v) sulfosalicylic acid (SSA) containing 0.2 mM EDTA was prepared for use with blanks, standards and samples. A solution of 9% SSA containing 0.2 mM EDTA was prepared for acidifying and thus stabilizing sulfhydryls in plasma samples. A 7 M ortho-phosphoric acid solution was prepared by mixing equal volumes of 85% (w/v) H_3PO_4 and water.

2.2.6.3. Standards

Calibrators and internal standards were made up in the 5% SSA solution containing 0.2 mM EDTA from 100 mM stock solutions of each compound. The calibrating compounds consisted of cysteine, glutathione and homocysteine, with an internal standard of penicillamine. The high calibrator consisted of 25 μM cysteine, 100 μM glutathione and 45 μM homocysteine. The low calibrator consisted of 2.5 μM cysteine, 10 μM glutathione and 4.5 μM homocysteine. The internal standard was prepared as a stock solution of 100 mM.

2.2.6.4. Derivatization and sample preparation

Samples used for the assay of sulfhydryl amino acids and peptides included plasma, kidney, liver, and left ventricular tissue. Equal volumes of plasma and 9% sulfosalicylic acid containing 0.2 mM EDTA provided plasma antioxidant stabilization. The protein precipitate was removed by microcentrifugation for 5 minutes and the supernatant was removed and stored at -80°C until assayed. Tissue samples were washed and quick frozen in liquid nitrogen immediately after being excised. Frozen tissues were then processed in a frozen state in a mikro-dismembrator and reconstituted 1:30 with 5% SSA containing 0.2 mM EDTA. The reconstituted tissue samples were sonicated at 40 hertz twice for 5 seconds, then once for 10 seconds. The samples were then centrifuged at 12,000 rpm for 20 minutes at 4°C, after which the supernatant was collected and kept on ice until assayed or stored at -80°C for up to one week.

The reaction mixtures for the determination of free reduced sulfhydryls consisted of 250 μL of TRIS buffer, pH 8.9, 65 μL of standard/sample, 10 μL of internal standard, 10 μL of ddH_2O and 175 μL of DTNB. Once all reagents and samples were added to the tubes, the mixture was allowed to sit for 5 minutes while the DTNB reacts with the proteins. The reaction

mixture was then re-acidified by adding 23 μL of 7 M H_3PO_4 dropwise while mixing to prevent protein precipitation. The derivatives were stable for at least 28 hours at room temperature.

The reaction mixture for the analysis of total sulfhydryls and reduced disulfides consisted of 250 μL of TRIS buffer, pH 8.9, 65 μL of standard/sample, 10 μL of internal standard, 175 μL of DTNB and 10 μL of DTT. The reaction mixture was incubated for 5 min and acidified as described for the reduced sulfhydryls. Once both reaction mixtures were ready, 130 μL was loaded into vials in duplicate and placed into the auto-sampler for injection onto the HPLC.

2.2.6.5. Chromatography

Chromatography of the sulfhydryl-DTNB and -DTT derivatives were accomplished using isocratic elution on a Supelco LC-18T column (150 x 4.6 mm, 3 μm) at 37°C using a waterbath. The mobile phase A was run at a flow rate of 0.9 ml/min. Sulfhydryl-derivatives were detected by UV absorbance at 345 nm. After 10 minutes of isocratic elution the mobile phase was switched to mobile phase B and ran for 8 minutes to elute excess DTNB or DTT reagent from the column. The column was then equilibrated for 7 minutes with mobile phase A before ejection of the next sample. Injection volume for each sample/standard was 50 μL .

2.2.6.6. Protein determination

Total protein concentrations were determined by the Bradford assay (1976) using reagents supplied by Bio-Rad and bovine serum albumin (BSA) as a standard. In a 1 mL disposable cuvette 20 μL of supernatant or serum samples were diluted to a total volume of 800 μL with distilled water (ddH_2O). Then 200 μL of Bio-Rad reagent was added to achieve a final volume of 1 mL, which was incubated at room temperature for 10 minutes. Following incubation, the absorbance was read at 595 nm in a spectrophotometer.

2.2.7. Malondialdehyde (MDA) Assay

The amount of membrane lipid peroxidation was determined using a spectrophotometric method adapted from Ohkawa, Ohishi and Yagi (1979). This method measures the interaction between thiobarbituric acid (TBA) and a breakdown product of lipid peroxidation, malondialdehyde (MDA).

2.2.7.1. Tissue and serum preparation

Rat tissues (kidney, liver, LV, mesentery and aorta) and serum samples were assayed for MDA concentrations. Tissues samples were rapidly homogenized in a phosphate buffer solution

immediately following their excise. The homogenates were centrifuged at 4°C, at 3000 rpm for 10 minutes, and the resultant supernatant was collected and stored at -80°C until assayed.

For serum samples, 2 ml of arterial blood were collected in labeled serum vacutainer tubes. The blood was stored at 4°C, left to clot for no more than 6 hours, and then centrifuged at 3000 rpm for 15 min. Serum was pipetted into labeled Eppendorf tubes and stored at -80°C until assayed.

2.2.7.2. Reagent preparation

A 100 μ M stock solution of MDA standard of tetraethoxypropane (TEP) was prepared by adding 6 μ L of TEP to 250 ml of ddH₂O. The resultant stock solution was then diluted to a 10 μ M working standard prior to assay by diluting 0.1 mL of the stock standard to 0.9 mL of ddH₂O. The MDA standard was stored under refrigeration for up to one week.

A solution of 8.1% (w/v) sodium dodecyl sulphate (SDS) was prepared by dissolving 8.1 g of SDS in 100 mL of ddH₂O. The SDS solution was stored at room temperature.

The solution of 0.67% (w/v) thiobarbituric acid (TBA) was prepared by dissolving 3.35 g of TBA in 40 mL of 2 M sodium hydroxide and pH was corrected to 5.9 with 0.1 M NaOH. The solution was then brought up to a 500 mL volume with ddH₂O.

Glacial acetic acid (HAc) was diluted with ddH₂O 1:4 in a fume hood, to make a 20% solution, and pH corrected to 3.4 with 0.1 M NaOH.

The 15:1 butanol/pyridine solution was prepared in a fume hood by combining 50 mL of pyridine with 750 mL of butanol.

2.2.7.3. Reaction and detection methods

The reaction mixture contained 50:50, 20% HAc, pH 3.4: 0.67% (w/v) TBA. Tissue supernatant and serum samples of 100 μ L were placed into glass test tubes mixed with 3 mL of reaction mixture and 200 μ L of 8.1% (w/v) SDS and brought to 1 mL volume with 700 μ L ddH₂O. Test tubes were incubated in a glycol bath at 98°C for one hour. After heating, 1.0 mL of ddH₂O was added to cool the reaction mixture, and 5.0 mL of butanol pyridine (15:1) was added to all tubes. Tubes were then capped and vortexed for 10 seconds each. The vortexed tubes were centrifuged at 3000 rpm for 15 minutes. The top layer was removed and read in a P2000 fluorometer at an excitation of 515 nm and emission of 553 nm. Concentrations of serum MDA were expressed as nmol/L, while tissue MDA was expressed as nM/mg protein, as compared to standards of 10 μ mol/L tetraethoxypropane.

2.2.8. Fasting Plasma Homocysteine Determination

2.2.8.1. Animal Fasting

All animals were fasted overnight, between 8 – 14 hours prior to surgery and bleeding, from either control or high-methionine diet depending on the experimental group.

2.2.8.2. Plasma preparation

Arterial blood was drawn and collected in EDTA vacutainer tubes on ice. Tubes were kept on ice and transported to blood chemistry laboratory at the Royal University Hospital in Saskatoon, Saskatchewan for the assay of total homocysteine concentrations.

2.2.8.3. Assay for the detection of Homocysteine in plasma

An Abbott IMx instrument utilizing fluorescence polarization (FPIA) technology was used to quantify total plasma homocysteine concentrations. A minimum of 50 µL of plasma was required to run the assay. The basic properties of the assay involve the reduction of homocysteine, mixed disulfides, and protein bound forms of homocysteine in the sample to form free homocysteine under the influence of the enzyme dithiothreitol (DTT). The total free Hcy is then transformed to S-adenosyl-L-homocysteine (SAH) by the enzyme SAH hydrolase and residual adenosine. Both the SAH form of homocysteine and the labeled S-adenosyl-L-cysteine fluorescein tracer, acting as antigens, compete for sites on an Anti-S-adenosyl-L-homocysteine mouse monoclonal antibody molecule (anti-S-adenosyl-L-homocysteine mouse monoclonal antibody, S-adenosyl-L-cysteine fluorescein tracer and SAH hydrolase are all contained in the homocysteine reagent pack provide by Abbott Laboratories). The polarization from the fluorescein-antigen conjugate was determined by its rate of rotation during the lifetime of the excited state in solution. The binding of the antigen to the antibody therefore changed the polarization of the compound. Lastly, the intensity of the polarization was measured by the FPIA optical assembly and related to the known antigen concentration (Shipchandler et al. 1995).

2.2.9. Statistical Methods

Results were expressed as mean \pm standard error of the mean (SEM). The data were tested by paired Student's t-tests. The difference was considered significant if p was less than 0.05.

3.0. RESULTS

3.1. CREATING A RAT MODEL WITH ELEVATED FASTING PLASMA HOMOCYSTEINE

Plasma total homocysteine concentrations measured in fasted WKY rats for control and high-methionine diet (MD) (5/group) are shown in Figure 3. Homocysteine concentrations were similar for the 2 and 8 week control rats and were 4.32 ± 0.16 and 4.93 ± 0.24 $\mu\text{mol/L}$ respectively.

At 2 weeks of MD exposure, mean total plasma homocysteine concentrations were 5.52 ± 0.29 $\mu\text{mol/L}$ and were significantly elevated compared to their age-matched controls ($p=0.01$), but not compared to the 8 week control group. After 4 and 8 weeks of MD exposure, plasma homocysteine levels were 5.89 ± 0.4 and 7.22 ± 0.24 $\mu\text{mol/L}$ respectively. Both exposure groups had significantly elevated homocysteine levels compared to the 2 week control rats ($p<0.05$). The homocysteine levels in the 8 week MD group were also significantly elevated compared to their age-matched controls ($p=0.01$).

A linear-regression analysis between total fasting plasma homocysteine and the duration of MD exposure is shown in Figure 4. Homocysteine concentrations beginning at 4.5 ± 0.01 $\mu\text{mol/L}$ increased linearly up to 7.2 ± 0.58 $\mu\text{mol/L}$ at 8 weeks MD exposure. As shown by the coefficient of determination ($r^2 = 0.9098$), 90% of the variance seen in homocysteine levels is due to the duration of exposure to MD. However, the observed changes in Hcy levels between 2, 4 and 8 weeks MD exposure groups failed to reach significance by student t-tests.

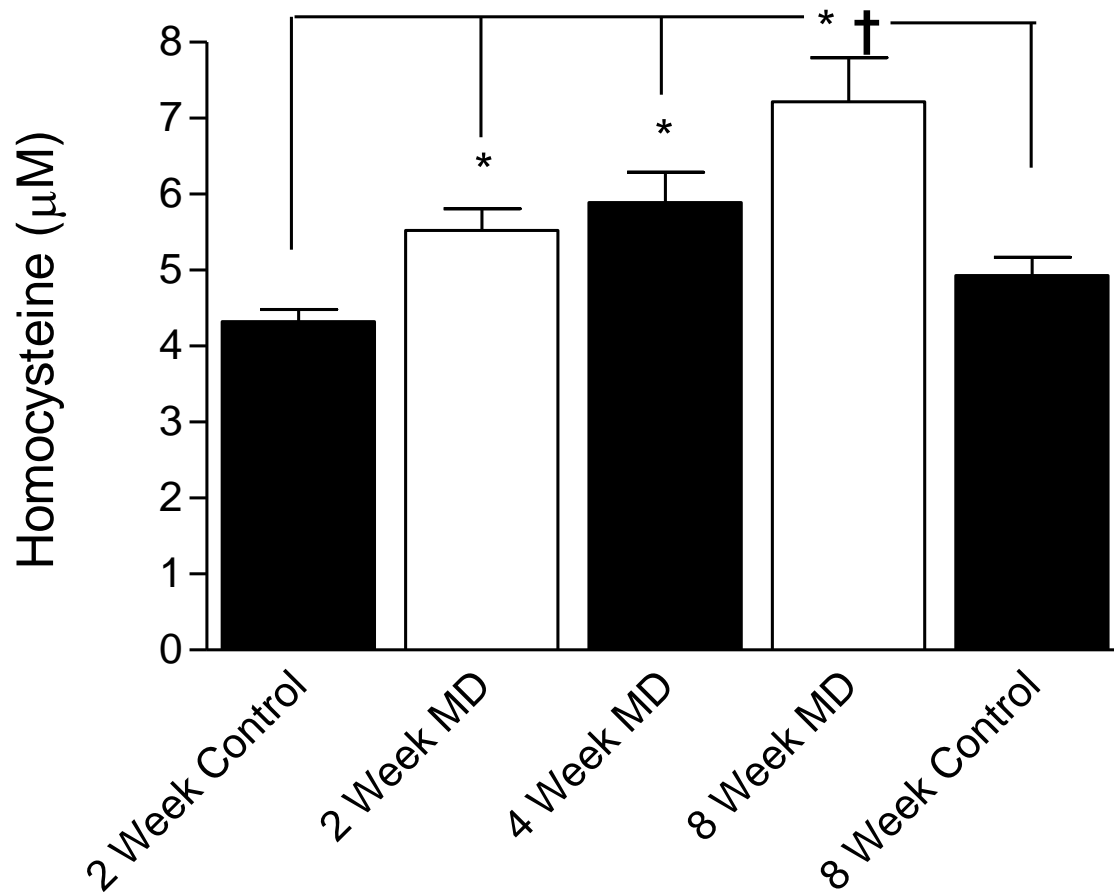


Figure 3 Plasma total homocysteine concentrations in fasted male WKY rats fed a modified diet containing 2% methionine for up to 8 weeks. Homocysteine levels were significantly increased by 2 weeks on the modified diet and remained elevated for the duration of diet exposure. Data is expressed as mean \pm SE (n = 4 – 5). * $p < 0.05$ vs. 2 week control; † $p < 0.05$ vs. 8 week control.

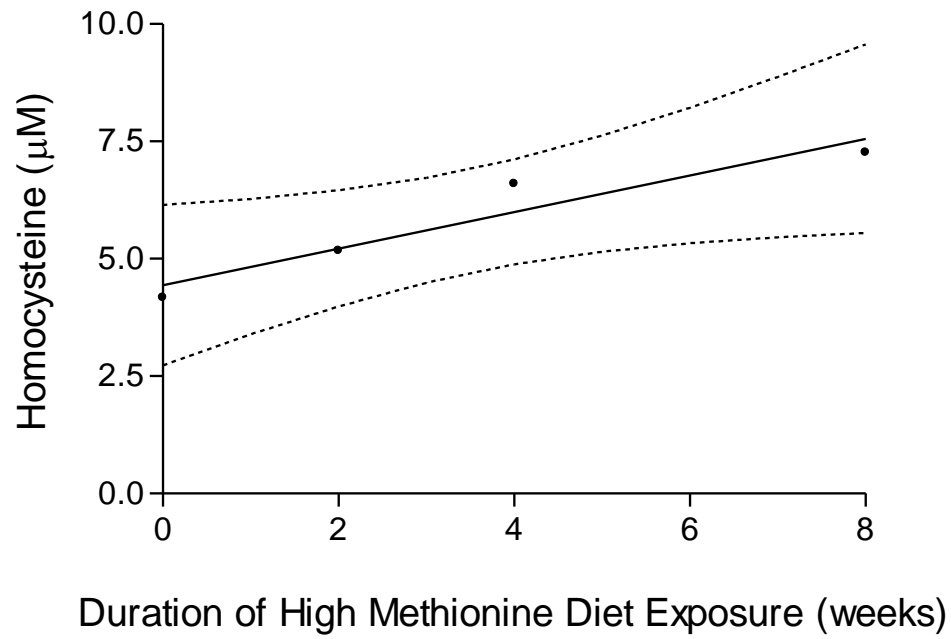


Figure 4 Linear regression analysis of fasting total plasma homocysteine concentrations contained in a 95% confidence interval (dotted lines) vs. duration of MD exposure in WKY rats ($n = 4 - 5$). Homocysteine concentrations increase linearly with duration of MD exposure ($r^2 = 0.9098$).

3.2. EFFECT OF HALOTHANE OR ISOFLURANE ANESTHETIC ON HEMODYNAMICS IN SPRAGUE DAWLEY RATS ON A HIGH METHIONINE DIET FOR UP TO 4 WEEKS

Since supplies of halothane were discontinued, the effect of different anesthetics on the hemodynamics of male SD rats was investigated. Groups were halothane and isoflurane anesthetic in control diet groups, 2 week MD exposure groups and 4 week MD exposure groups. The following hemodynamic parameters: left ventricular systolic pressure, left ventricular end-diastolic pressure, left ventricular developed pressure, myocardial indices of contractility and relaxation, arterial systolic pressure, diastolic pressure and mean arterial pressure.

3.2.1. Left ventricular systolic pressure (LVSP)

The effect of anesthetic and MD exposure on LVSP is shown in Figure 5. LVSP is significantly elevated ($p=0.014$) in the isoflurane control (104.96 ± 2.73 mmHg) compared to the halothane control group (94.37 ± 1.46 mmHg). The reverse was seen at the 2-week diet exposure period where there was a trend towards an increase in LVSP in the halothane group where the 2-week halothane group LVSP was 105.75 ± 2.59 mmHg compared to in the 2-week isoflurane diet group 94.82 ± 3.98 mmHg ($p=0.050$). Increased exposure periods to the MD increased LVSP in the halothane anesthetized rats only. The 2-week diet exposure group, anesthetized with halothane, had significantly higher LVSP than their anesthetic control ($p=0.0065$) as did the 4-week diet exposure halothane group (116.87 ± 5.02 mmHg), ($p=0.0077$). The LVSP in the 4-week diet exposed halothane group (109.28 ± 2.96 mmHg) was significantly greater than the 2-week isoflurane diet group ($p=0.0074$), but not the 4-week isoflurane diet group ($p=0.23$).

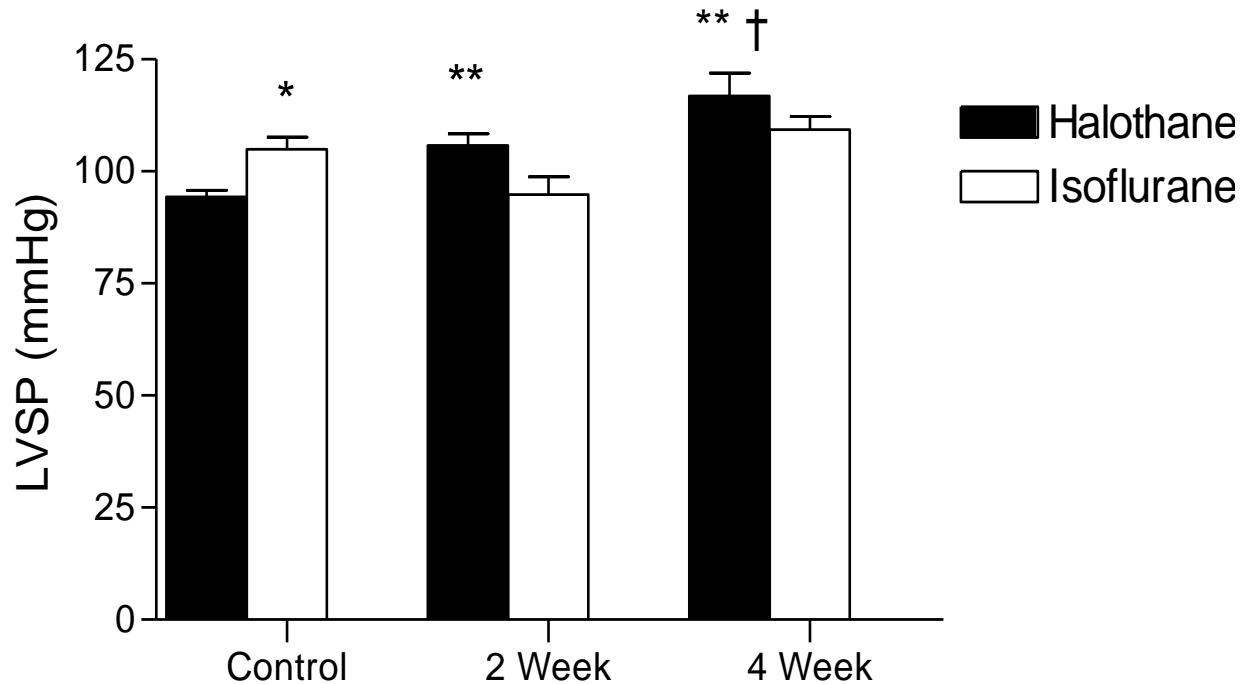


Figure 5 Left ventricular systolic pressure in control or MD exposed SD rats for 2 and 4 weeks anesthetized with halothane or isoflurane. Isoflurane control LVSP was significantly elevated as compared to halothane controls ($p=0.014$). LVSP was significantly elevated in the 2-week and 4-week halothane MD exposed groups compared to halothane controls. The 4-week MD group with halothane anesthetic was also found to be significantly elevated above the 2-week isoflurane MD exposed group. Data is expressed as mean \pm SE ($n = 5 - 6$). * $p=0.0141$ vs. halothane control. ** $p<0.01$ vs. halothane control. † $p=0.0074$ vs. I2.

3.2.2. Left ventricular end-diastolic pressure (LVEDP)

The effect of anesthetic and MD exposure on left ventricular end-diastolic pressure is shown in Figure 6. There was no significant change in LVEDP seen with anesthetic use or with diet exposure between all groups. The LVEDPs in halothane control, 2-week and 4-week high methionine exposure groups were 15.65 ± 2.63 , 14.77 ± 1.31 and 18.85 ± 3.26 mmHg respectively. In isoflurane control, 2-week and 4-week high methionine exposure groups, LVEDPs were 8.78 ± 3.37 , 11.20 ± 1.30 and 11.53 ± 4.08 mmHg respectively.

3.2.3. Left ventricular developed pressure (LVDP)

The effect of anesthetic and MD exposure on left ventricular developed pressure is shown in Figure 7. LVDP were 83.25 ± 3.23 and 101.4 ± 4.62 mmHg for the halothane and isoflurane controls respectively, and were significantly higher with isoflurane treatment ($p = 0.02$). At 4 weeks of MD exposure, LVDP for the halothane and isoflurane groups were 97.67 ± 3.39 and 107.60 ± 1.06 mmHg respectively and were significantly higher with isoflurane treatment ($p=0.038$). The LVDP in the 4 week halothane MD exposed group was significantly elevated vs. its anesthetic control ($p=0.013$).

Within the isoflurane groups there was no significant differences observed in LVSP. There were also no differences observed between the 2 weeks MD exposure groups and any other MD or anesthetic groups.

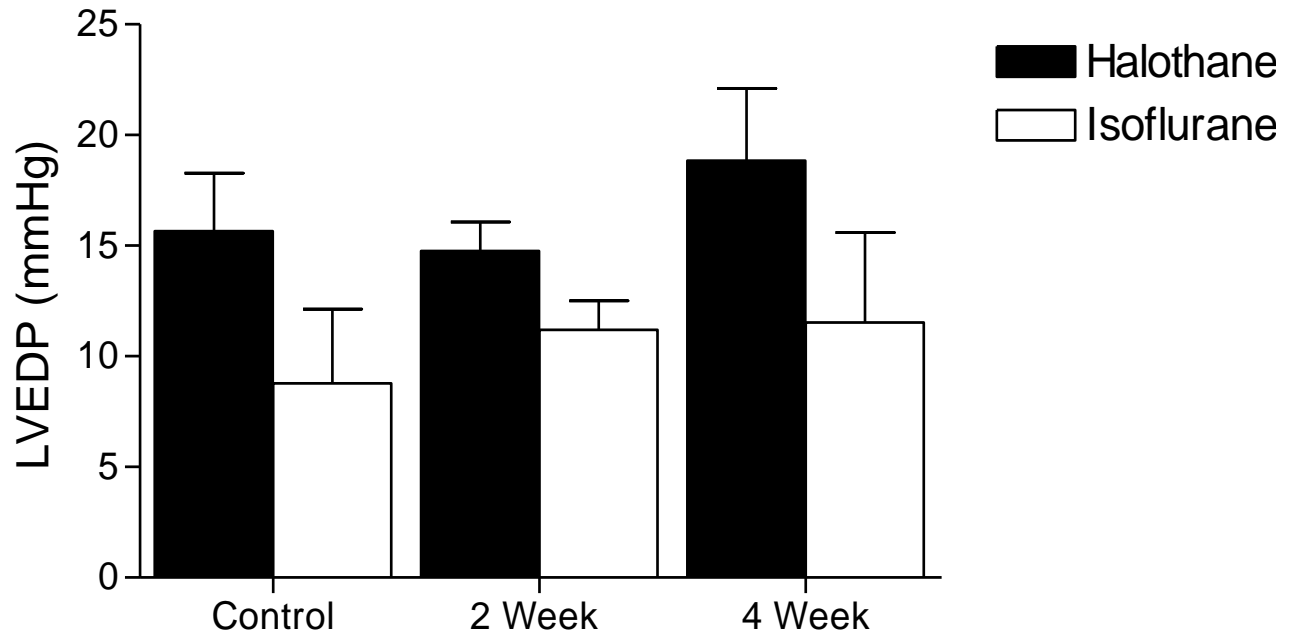


Figure 6 Left ventricular end-diastolic pressure (LVEDP) in control or MD exposed SD rats for 2 and 4 weeks anesthetized with halothane or isoflurane. There was no significant change in LVEDP measured between any of the anesthetic or diet exposure groups. Data is expressed as mean \pm SE (n = 5 – 6).

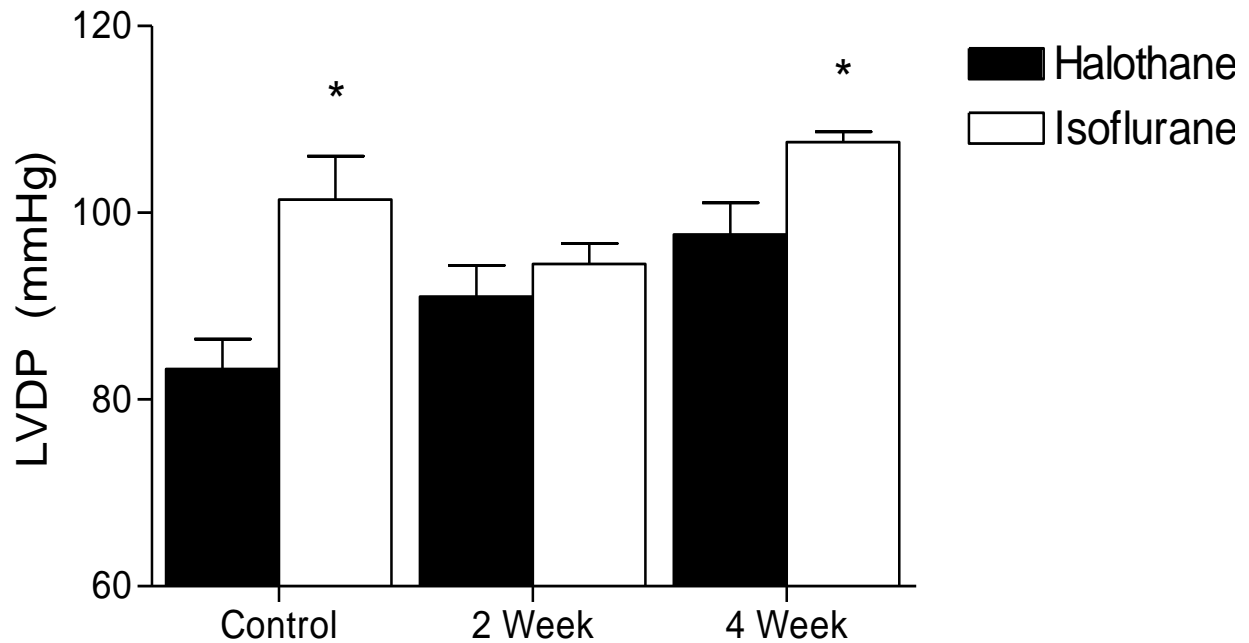


Figure 7 Left ventricular developed pressure (LVDP) in control or MD exposed SD rats for 2 and 4 weeks and anesthetized with halothane or isoflurane. LVDP was measured as the maximum LV pressure – minimum LV pressure. Both the isoflurane control and 4 week isoflurane MD exposed rats had significantly higher LVDP than their respective matched halothane control and 4 week MD exposed rats. Data is expressed as mean \pm SE (n = 5 – 6). * p<0.05.

3.2.4. Myocardial index of contractility (+ dp/dt) and relaxation (- dp/dt)

The effect of anesthetic and MD exposure on the myocardial index of contractility is shown in Figure 8. Halothane anesthesia resulted in significantly lower + dp/dt compared to isoflurane anesthesia. The + dp/dt for halothane vs. isoflurane groups was respectively 4119 ± 273 and 6885 ± 371 mmHg/sec, ($p=0.0095$) in the control groups, 4711 ± 300 and 6624 ± 372 mmHg/sec, ($p=0.017$) in the 2 week diet groups and 5090 ± 225 and 8076 ± 341 mmHg/sec, ($p=0.0043$) in the 4 week diet group.

The effect of anesthetic and MD exposure on the myocardial index of relaxation is also shown in Figure 8. No significant change in - dp/dt was observed between matched anesthetic groups. The - dp/dt in control halothane and isoflurane was 3627 ± 491 and 4299 ± 396 mmHg/sec respectively. In the 2 week diet treated groups, halothane and isoflurane groups the - dp/dt was 4356 ± 382 and 4376 ± 467 mmHg/sec respectively and in the 4 week diet group 4894 ± 270 and 5459 ± 193 mmHg/sec respectively. At 4 weeks of MD exposure, - dp/dt was significantly increased in each anesthetic group vs. their controls with p values of $p=0.035$ and $p=0.046$ for the halothane and isoflurane groups respectively.

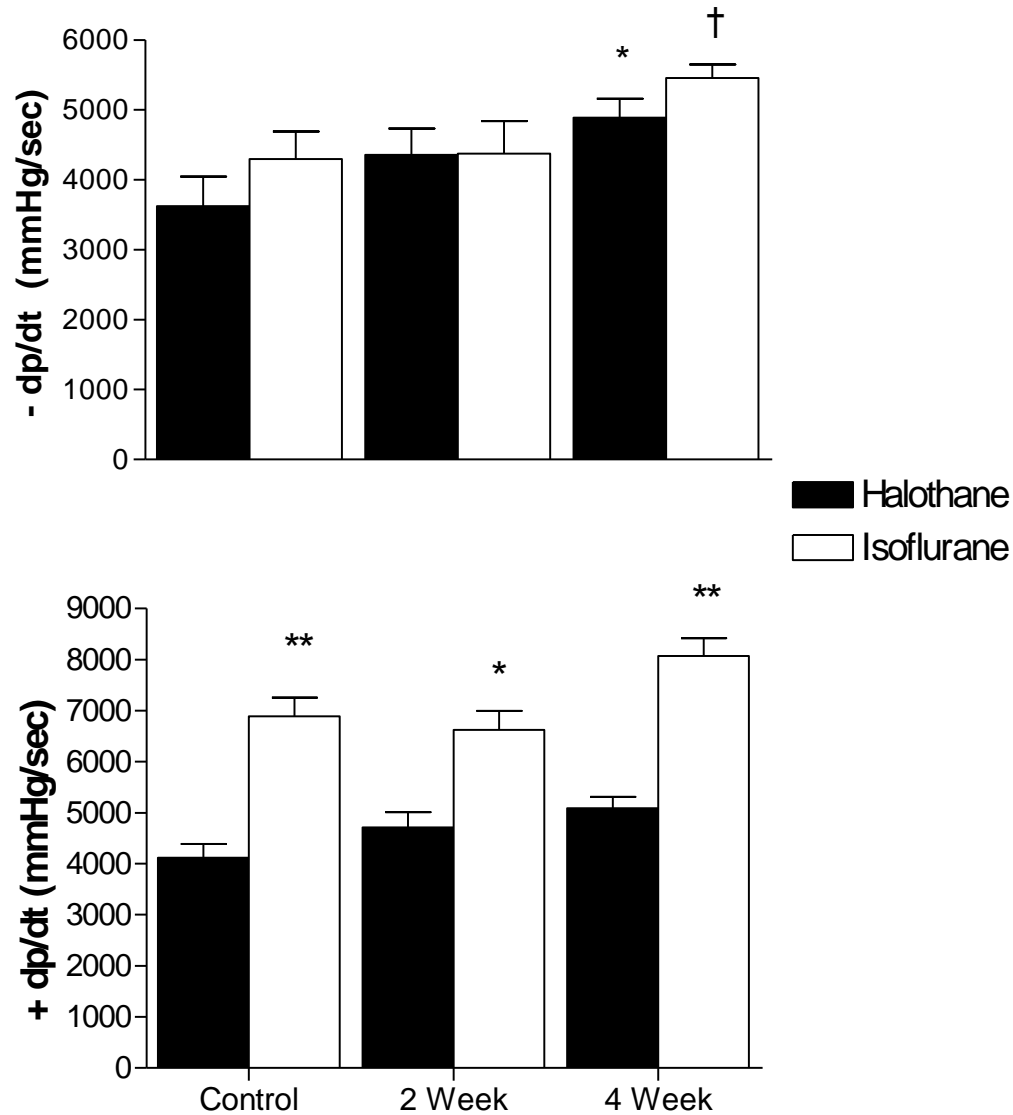


Figure 8 Myocardial index of contractility (+dp/dt) and relaxation (-dp/dt) in control or MD exposed SD rats for 2-4 weeks anesthetized with halothane or isoflurane. All of the isoflurane anesthetized animals had significantly higher +dp/dt than their respective matched halothane groups. The 4-week MD groups -dp/dt were significantly elevated as compared to their matched anesthetic control. Data is expressed as mean \pm SE; (n = 5 – 6). – dp/dt: * p=0.035 vs. halothane control, † p=0.046 vs. isoflurane control. +dp/dt: * p<0.05; ** p<0.01

3.2.5. Systolic Arterial Pressure (SP)

The effect of different anesthetics and MD exposure on systolic arterial pressure (SP) is shown in Figure 9. No significant changes were observed in SP between halothane and isoflurane anesthetic controls (97.77 ± 1.17 and 98.90 ± 5.54 mmHg, $p=0.85$ respectively), and 2-week diet exposure groups (104.4 ± 3.10 and 107.2 ± 3.97 mmHg, $p=0.43$ respectively). The SP of the 4-week halothane MD group (118.9 ± 4.12 mmHg) was significantly elevated compared to its anesthetic control ($p=0.0078$), the isoflurane control ($p=0.02$) and the 4-week diet isoflurane group (106.6 ± 2.3 mmHg; $p=0.041$). There was a trend towards a significant increase in SP in the 2-week MD halothane group compared to its anesthetic control ($p=0.052$).

3.2.6. Diastolic Arterial Pressure (DP)

The effect of different anesthetics and MD exposure on diastolic arterial pressure is shown in Figure 9. In the control groups, halothane treatment DP (81.52 ± 3.59 mmHg) was significantly higher ($p=0.02$) than isoflurane (67.65 ± 3.64 mmHg). Anesthetic treatment had no significant effect on DP between either the halothane or isoflurane 2-week (79.97 ± 2.54 and 73.07 ± 4.84 mmHg respectively, $p=0.39$) or 4-week diet groups (88.33 ± 4.83 and 75.33 ± 2.69 mmHg respectively, $p=0.065$). Diet treatment also produced no change between 2 and 4 week isoflurane ($p=0.48$ and $p=0.09$) and halothane ($p=0.48$ and $p=0.24$) MD groups vs. their controls.

3.2.7. Mean Arterial Pressure (MAP)

The effect of different anesthetics and MD exposure on mean arterial pressure is shown in Figure 9. There was a significant increase in MAP in the halothane control group, 84.89 ± 1.76 mmHg vs. the isoflurane control group, 74.62 ± 3.05 mmHg, $p=0.027$. There was no change observed between the halothane and isoflurane 2-week diet exposure groups MAP (85.52 ± 1.08 and 86.87 ± 3.79 mmHg respectively). The 4-week isoflurane diet exposure group MAP, 85.76 ± 2.55 mmHg was significantly elevated compared to its anesthetic control ($p=0.023$). There was a trend towards an increase in MAP with increased diet exposure as the 4-week halothane diet group MAP, 96.99 ± 5.10 mmHg was elevated, compared to 84.89 ± 1.76 mmHg in the halothane control ($p=0.066$).

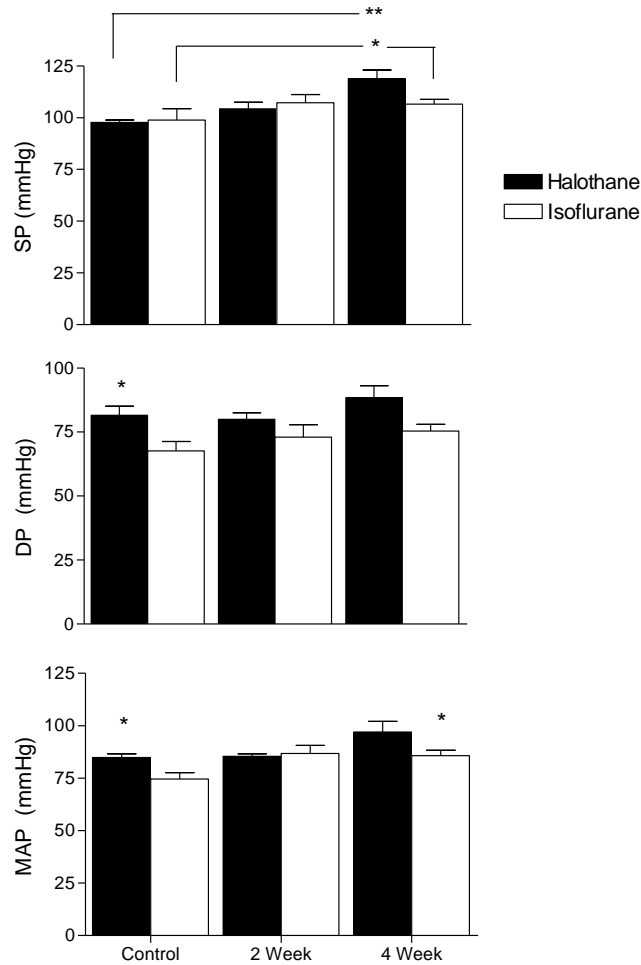


Figure 9 Arterial systolic pressure (SP), diastolic pressure (DP) and mean arterial pressure (MAP) measured in control or MD exposed SD rats for 2 and 4 weeks anesthetized with halothane or isoflurane. The DP in halothane anesthetized controls was significantly higher than the isoflurane anesthetized controls (* $p=0.02$). The SP in the 4 week MD halothane group was significantly higher than its anesthetic control (** $p=0.008$), the isoflurane control (* $p=0.019$) and the 4-week isoflurane diet exposure group (* $p=0.04$). The MAP in the halothane anesthetized control was significantly higher than the isoflurane anesthetized controls (* $p=0.027$). MAP was also significantly elevated in the 4-week isoflurane diet exposure group vs. its anesthetic control (* $p=0.023$). Data is expressed as mean \pm SE, $n = 5 - 6$.

3.3. HEMODYNAMICS OF RATS WITH ELEVATED PLASMA HOMOCYSTEINE LEVELS

A hemodynamic investigation was made on the effect of MD exposure in male WKY rats for up to 8 weeks, including 2-week and 8-week age-matched controls measured under isoflurane anesthetic. Hemodynamic parameters are divided into LVSP, LVEDP, LVDP, myocardial indices of contractility and relaxation, SP, DP and MAP.

3.3.1. Left ventricular systolic pressure (LVSP)

The effect of MD exposure on LVSP is shown in Figure 10. LVSP was significantly elevated by 6 weeks of MD feeding (115.2 ± 1.48 mmHg, $p=0.037$) and remained significantly elevated at 8-weeks of MD exposure (124.7 ± 5.0 mmHg, $p=0.0086$) compared to the 2-week control (98.86 ± 5.1 mmHg). The 4-week diet group (109.3 ± 2.96 mmHg) had a significantly higher LVSP than the 2-week diet group ($p=0.0194$), as did the 6-week ($p=0.003$) and 8-week ($p=0.0016$) diet groups. As well, the 8-week diet group had a significantly higher LVSP than the 4-week diet group ($p=0.0385$). The 8-week high methionine group was the only group found to have significantly elevated LVSP compared to the 8-week control group (110 ± 5.21 mmHg, $p=0.0243$).

3.3.2. Left ventricular end-diastolic pressure (LVEDP)

The effect of MD exposure on LVEDP is shown in Figure 11. LVEDP was significantly elevated in the 8-week MD group (20.06 ± 2.96 mmHg) compared to age-matched control (6.36 ± 1.61 mmHg, $p=0.0067$), as well as the 2-week diet (11.2 ± 1.3 mmHg, $p=0.0409$) and 6-week diet groups (9.9 ± 1.46 mmHg, $p=0.0277$). The LVEDP for the 2-week control, 10.58 ± 3.68 mmHg, and 4-week diet group, 11.53 ± 4.08 mmHg were not significantly different than any other group. Also the LVEDP in the 8-week control group was significantly elevated compared to the 2-week diet exposure groups ($p=0.048$).

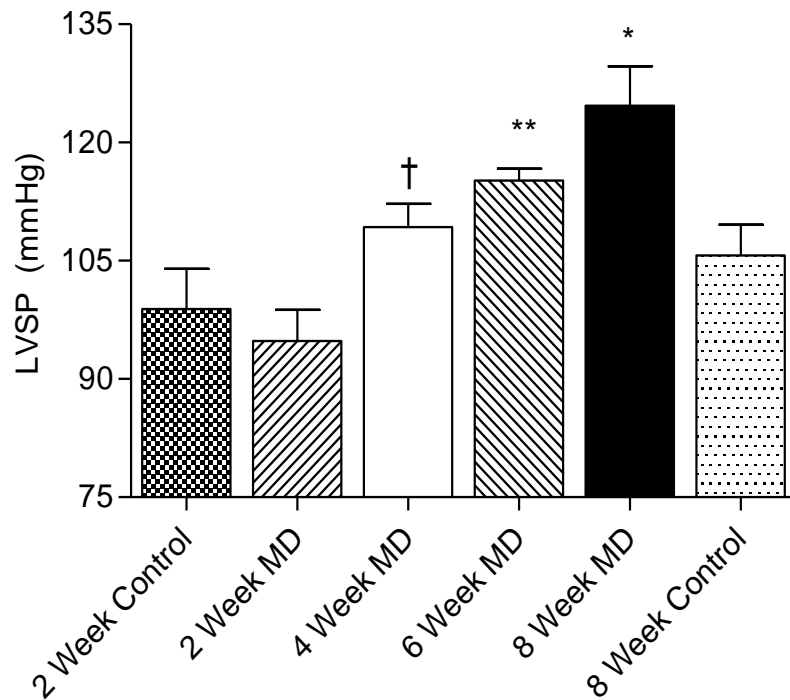


Figure 10 Left ventricular systolic pressure (LVSP) measured in male WKY rats fed a high methionine diet. Rats were divided into groups exposed to a high (2%) methionine diet (MD) for 2, 4, 6 and 8 weeks as well as 2 and 8 week age-matched WKY rats fed a control diet. LVSP was significantly elevated compared to the 2-week control group by 6 weeks of MD feeding. LVSP in the 8-week MD group was significantly elevated compared to the 2-week control, as well as the 2, 4-week diet groups and the 8-week control group. The 6-week diet group was also significantly elevated compared to the 2-week diet group. The 4-week diet group also had a significantly higher LVSP than the 2-week diet group. Data is expressed as mean \pm SE, n = 5 – 6. *: p=0.0086 vs. 2-week control; p=0.0016 vs. 2-week MD; p=0.0243 vs. 8-week control. **: p=0.0369 vs. 2-week control; p=0.003 vs. 2-week MD. †: p=0.0194 vs. 2-week MD.

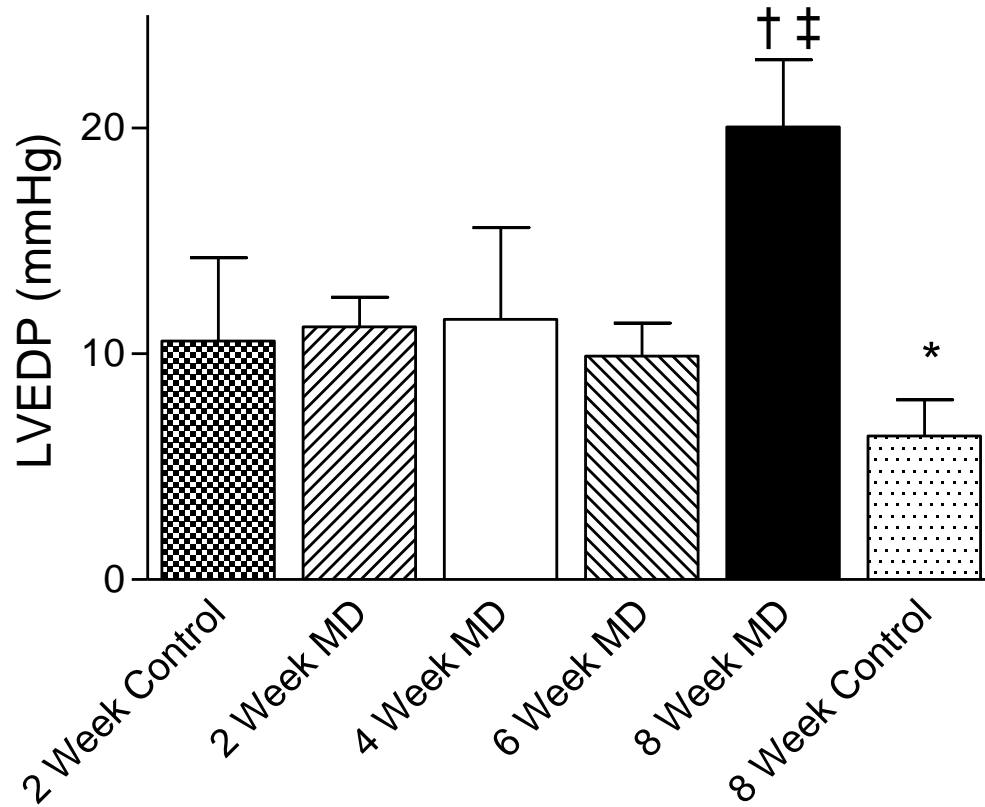


Figure 11 Left ventricular end-diastolic pressure (LVEDP) measured in male WKY rats fed a high methionine diet. Rats were divided into groups exposed to a high (2%) methionine diet (MD) for 2, 4, 6 and 8 weeks as well as 2 and 8 week age-matched WKY rats fed a control diet. LVEDP was significantly elevated in the 8 week MD compared to its age-matched control, and the 2-week and 6-week MD groups. The 8-week control group had a significantly lower LVEDP than the 2-week MD group. Data is expressed as mean \pm SE, $n = 5 - 6$. † $p < 0.05$ vs. 2 and 6-week MD; ‡ $p = 0.0067$ vs. 8-week control; * $p = 0.0478$ vs. 2-week MD.

3.3.3. Left ventricular developed pressure (LVDP)

The effect of MD exposure on LVDP is shown in Figure 12. The 4, 6 and 8-week diet groups and the 8-week control group all had significantly higher LVDP (100.3 ± 1.46 , 105.3 ± 1.3 , 104.6 ± 4.71 and 103.6 ± 3.68 mmHg respectively), than the 2-week diet exposure group (86.6 ± 2.01 mmHg) ($p < 0.01$). The 6-week diet group also had a significantly higher LVDP than the 4-week diet group and 2-week control LVDP (93.8 ± 4.13 mmHg) ($p < 0.05$).

3.3.4. Myocardial index of contractility (+dp/dt) and relaxation (- dp/dt)

The effect of MD exposure on the myocardial index of contractility (+dp/dt) is shown in Figure 13. The + dp/dt was significantly elevated after 4 and 6 weeks of MD exposure (8076 ± 340 and 8867 ± 328 mmHg/sec respectively) compared to the 2 week control group (6149 ± 791 mmHg/sec, $p = 0.016$ vs. 4 week MD and $p = 0.0043$ vs. 6 week MD) and the 2 week MD group (6031 ± 666 mmHg/sec, $p = 0.0087$ vs. 4 week MD and $p = 0.0022$ vs. 6 week MD). After 8 weeks of MD exposure +dp/dt significantly decreased (7009 ± 506 mmHg/sec) compared to the 6 week diet exposure group ($p = 0.018$) as well as the 8 week control group (8831 ± 160 mmHg/sec, $p = 0.026$). The +dp/dt was also significantly elevated in the 8 week control group compared to 2 week controls ($p = 0.016$).

The effect of MD exposure on the myocardial index of relaxation (-dp/dt) is shown in Figure 14. The -dp/dt in the 4 and 6 week MD exposure groups (5459 ± 193 and 6525 ± 463 mmHg/sec, respectively) was significantly higher than the 2 week control group (4299 ± 396 mmHg/sec; $p = 0.0317$ vs. 4 week MD and $p = 0.0081$ vs. 6 week MD). The 8 week control group -dp/dt (5854 ± 451 mmHg/sec) was significantly elevated compared to the 2 week controls ($p = 0.0360$). Exposure for 6 weeks on the MD showed significantly increased -dp/dt compared to 2 weeks MD (4376 ± 467 mmHg/sec, $p = 0.0360$).

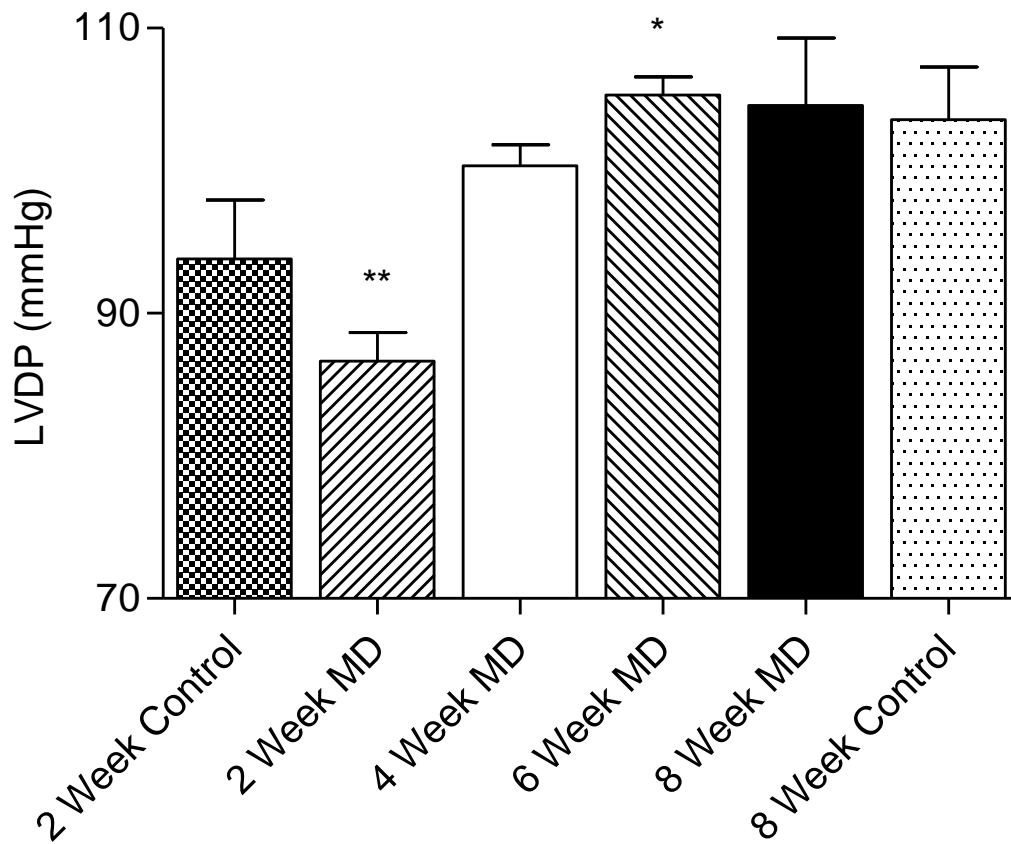


Figure 12 Left ventricular developed pressure (LVDP) measured in male WKY rats fed a high methionine diet. Rats were divided into groups exposed to a high (2%) methionine diet (MD) for 2, 4, 6 and 8 weeks as well as 2 and 8 week age-matched WKY rats fed a control diet. The LVDP in the 2-week MD group was significantly less than the 4, 6 and 8-week MD groups as well as the 8 week control group. The 6-week MD group also had significantly higher LVDP than the 2 week control and 4-week MD groups. Data is expressed as mean \pm SE, $n = 5 - 6$. **: $p=0.0079$ vs. 4 and 8-week MD; $p=0.0043$ vs. 6-week MD; $p=0.0067$ vs. 8-week control. *: $p=0.0452$ vs. 2-week control; $p=0.035$ vs. 4-week MD.

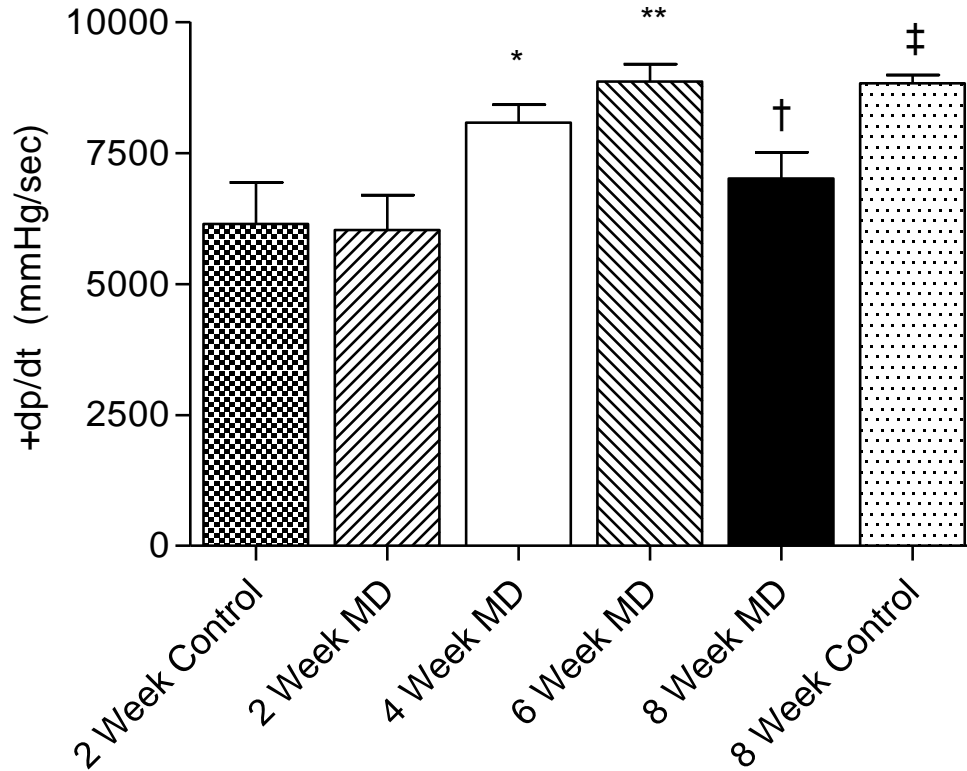


Figure 13 Myocardial index of contractility (+dp/dt) measured in male WKY rats fed a high methionine diet. Rats are divided into groups exposed to MD for 2, 4, 6 and 8 weeks as well as 2 and 8 week age-matched WKY rats fed a control diet. The +dp/dt was significantly elevated in the 8 week compared to the 2 week control groups. The +dp/dt was also significantly elevated in the 4 and 6 week MD groups compared to the 2-week control and the 2 week MD group. At 8 weeks MD exposure, + dp/dt decreased significantly compared to the 6 week MD group and the 8 week control group. Data is expressed as mean \pm SE. *: p=0.016 vs. 2 week control, p=0.0087 vs. 2 week MD; **: p=0.0043 vs. 2 week control, p=0.0022 vs. 2 week MD; †: p=0.0179 vs. 6 week MD; ‡: p=0.016 vs. 2 week control.

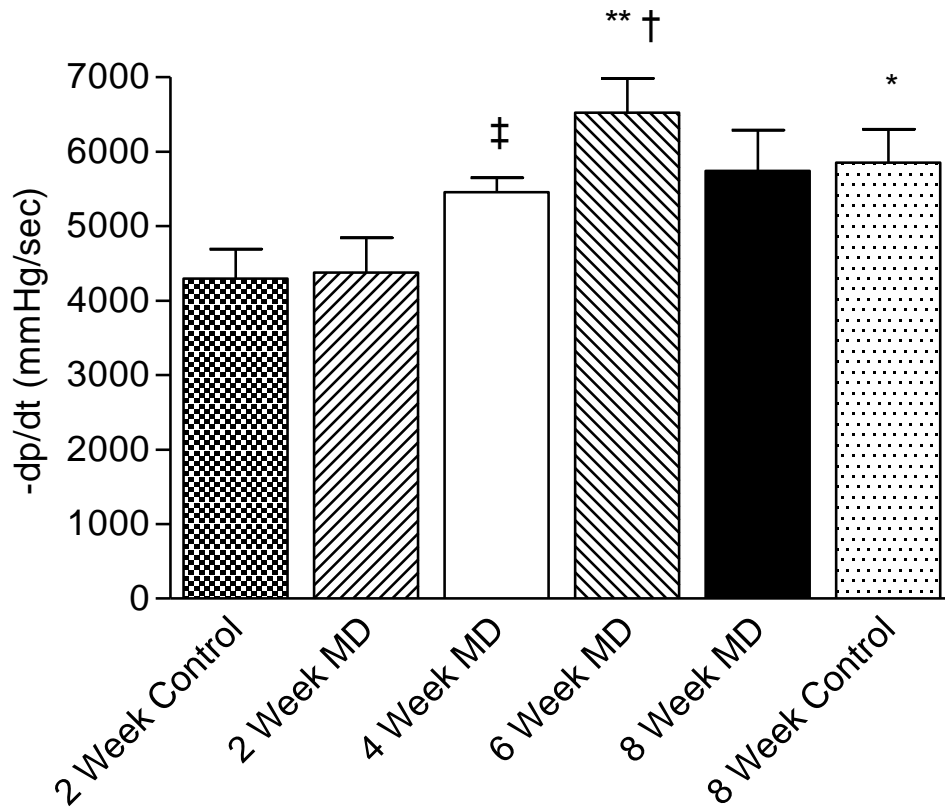


Figure 14 Myocardial index of relaxation ($-\text{dp}/\text{dt}$) measured in male WKY rats fed a high methionine diet. Rats are divided into groups exposed to MD for 2, 4, 6 and 8 weeks as well as 2 and 8 week age-matched WKY rats fed a control diet. The $-\text{dp}/\text{dt}$ for the 8 week controls was significantly higher than the 2 week controls. Also, the $-\text{dp}/\text{dt}$ was significantly elevated in the 4 and 6 week MD groups compared to the 2 week controls. The 6 week but not the 4 week MD group $-\text{dp}/\text{dt}$ was significantly greater than the 2 week MD group. Data is expressed as mean \pm SE. *: $p=0.036$ vs. 2 week control; **: $p=0.0081$ vs. 2 week control; †: $p=0.036$ vs. 2 week MD; ‡: $p=0.032$ vs. 2 week control.

3.3.5. Arterial Pressure

The effects of MD exposure SP, DP and MAP are shown in Figure 15. All measures of arterial pressure (MAP, SP and DP) were significantly elevated in the 8 week MD group (107 ± 5.2 , 126 ± 6.0 , and 97.2 ± 5.0 mmHg, respectively) compared to all other groups, except SP in the 6 week MD group. Data and p values were: 2 week control group, MAP: 78.1 ± 4.2 mmHg, $p=0.0027$; SP: 98.9 ± 5.5 mmHg, $p=0.0094$; DP: 67.7 ± 3.6 mmHg, $p=0.0020$; the 2 week MD group, MAP: 82.9 ± 5.0 mmHg, $p=0.011$; SP: 103 ± 5.6 mmHg, $p=0.019$; DP: 73.1 ± 4.8 mmHg, $p=0.0084$; and the 4 week MD group, MAP: 85.8 ± 2.5 mmHg, $p=0.015$; SP: 107 ± 2.4 mmHg, $p=0.026$; DP: 74.3 ± 2.7 mmHg, $p=0.0082$. MAP and DP of the 6 week MD group (MAP: 91.0 ± 2.4 mmHg, $p=0.0387$; DP: 81.0 ± 3.0 mmHg, $p=0.0318$) were significantly different than the 8 week MD group. The arterial pressures of the 8 week control group (MAP: 88.9 ± 3.7 mmHg, $p=0.0257$; SP: 107 ± 4.2 mmHg, $p=0.0324$; DP: 79.8 ± 3.4 mmHg, $p=0.0238$) were also significantly different than the 8 week MD group.

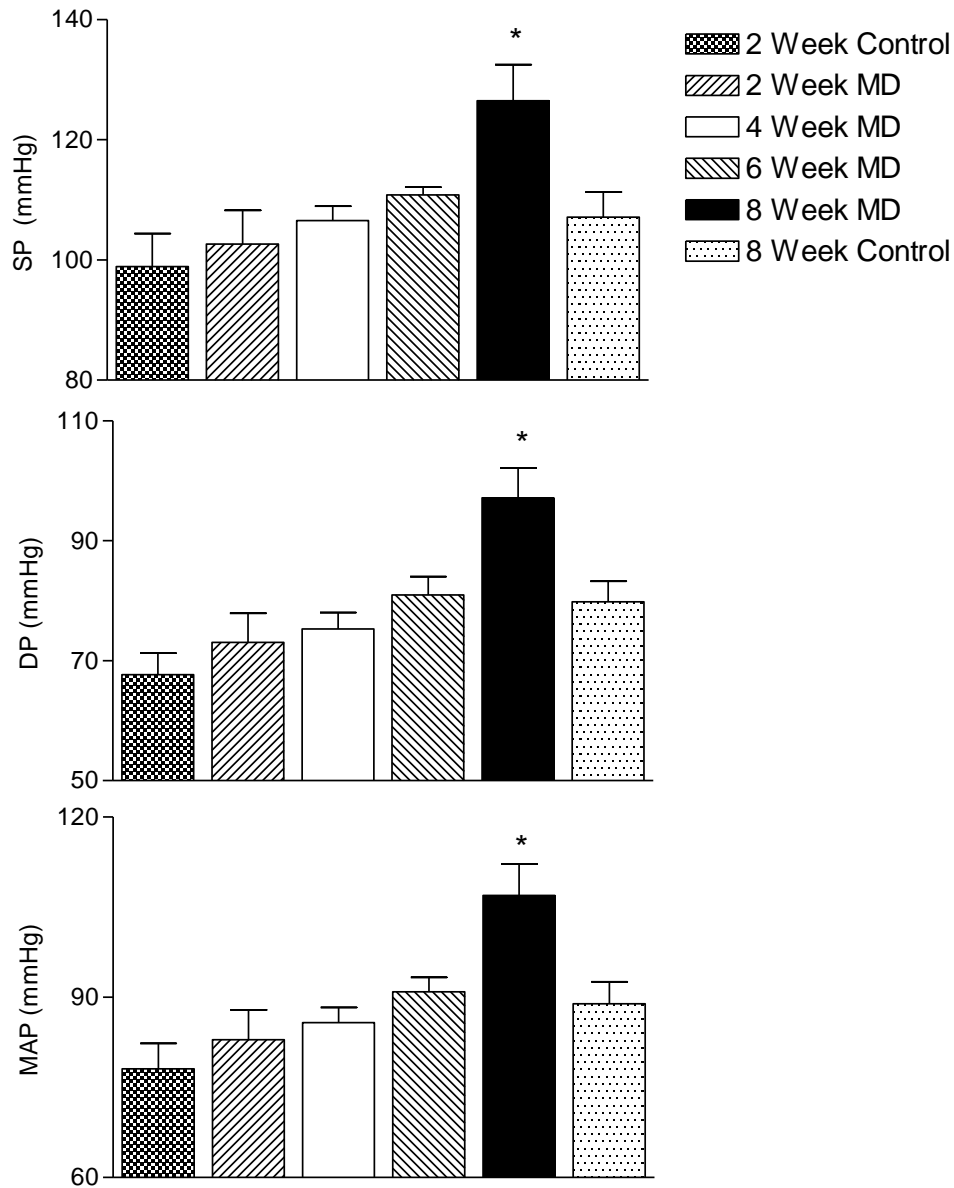


Figure 15 Systolic pressure (SP), diastolic pressure (DP) and mean arterial pressure (MAP) measured in male WKY rats fed a high methionine diet. Rats are divided into groups exposed to MD for 2, 4, 6 and 8 weeks as well as 2 and 8 week age-matched WKY rats fed a control diet. All arterial pressures of the 8-week MD rats were significantly elevated compared to all groups, except SP in the 6 week MD group ($p=0.062$). Data is expressed as mean \pm SE. $n = 5 - 6$. * $p < 0.05$ vs. all groups; except SP in the 6 week MD.

3.4. EFFECT OF ORAL HEPARIN ON HEMODYNAMICS AND FASTING PLASMA HOMOCYSTEINE CONCENTRATIONS IN HIGH METHIONINE DIET FED WISTAR KYOTO RATS FOR 8 WEEKS

The WKY rats were divided into a MD exposed group, MD plus oral heparin (1mg/kg/48hr) group for 8 weeks and age-matched controls. Hemodynamic measurements were performed under isoflurane anesthesia.

3.4.1. Fasting Plasma Homocysteine Levels

The effect of oral heparin treatment in WKY rats fed MD on total fasting plasma homocysteine concentrations is shown in Figure 16. Fasting plasma homocysteine concentrations were significantly elevated after 8 weeks of MD feeding ($7.17 \pm 0.46 \mu\text{mol/L}$) compared to their age-matched control ($5.46 \pm 0.36 \mu\text{mol/L}$, $p=0.0097$). Homocysteine concentrations remained elevated in the oral heparin treated MD group ($7.02 \pm 0.40 \mu\text{mol/L}$) compared to the normal diet age-matched controls ($p=0.0170$). Oral heparin treatment had no effect on fasting plasma homocysteine concentrations in rats fed the MD.

The effect of oral heparin treatment in WKY rats fed MD on total body mass was recorded prior to surgery as shown in Figure 17. There were no differences observed in total body mass between the control ($309 \pm 9.6 \text{ g}$) and MD diet groups with or without oral heparin treatment ($301 \pm 9.9 \text{ g}$; $315 \pm 5.4 \text{ g}$) respectively, suggesting that each animal was fed and consumed relatively the same amount of the MD.

3.4.2. Left Ventricular Systolic Pressure (LVSP)

The effect of oral heparin treatment on LVSP of MD fed WKY rats is shown in Figure 18. LVSP was significantly elevated in the MD group ($122.6 \pm 3.2 \text{ mmHg}$) compared to the age-matched control ($112.3 \pm 2.9 \text{ mmHg}$, $p=0.0315$). There was no significant difference observed in LVSP between the oral heparin treated group ($119.9 \pm 3.2 \text{ mmHg}$) and all other groups, suggesting oral heparin treatment attenuated the increase seen in LVSP by MD feeding.

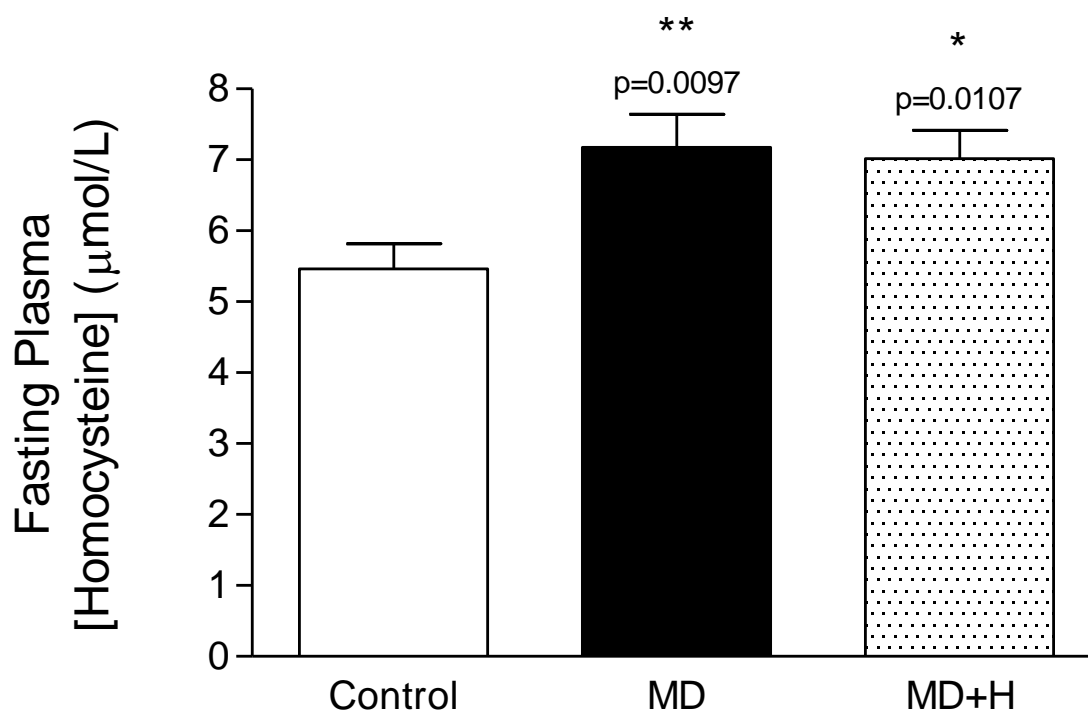


Figure 16 Effect of oral heparin (H) (1 mg/kg/48hours), given by gavage, on plasma total homocysteine concentrations in male WKY rats fed a 2% methionine diet (MD) for 8 weeks. Homocysteine levels were significantly elevated in MD fed rats after 8 weeks and remained elevated in the MD plus oral heparin group compared to age-matched controls. Data is expressed as mean \pm SE (n = 5). * p=0.0107; ** p=0.0097 vs. control.

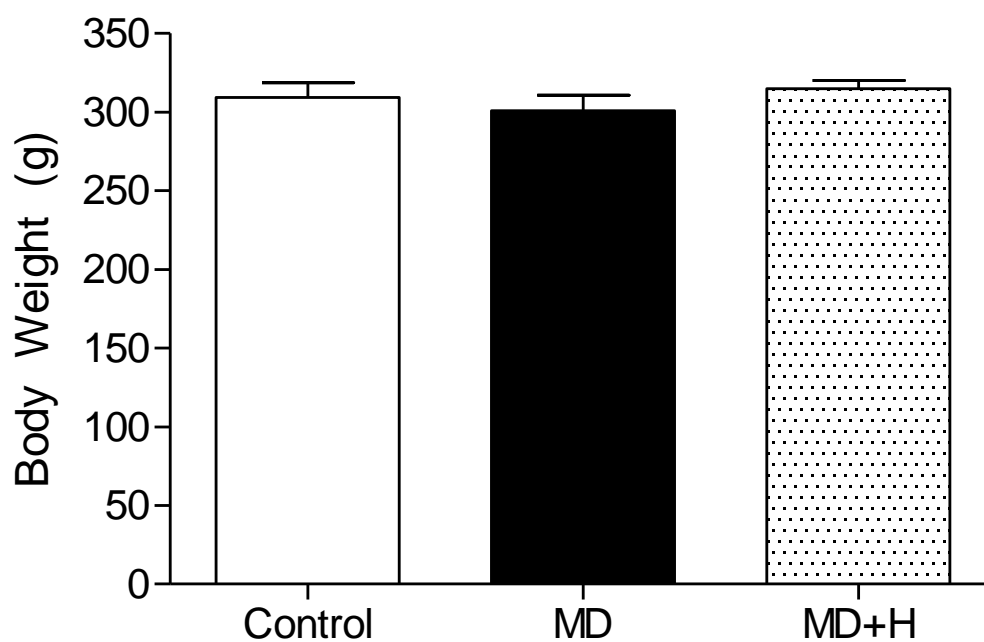


Figure 17 Effect of oral heparin (H) (1 mg/kg/48hours), given by gavage, on body mass in male WKY rats fed a 2% methionine diet (MD) for 8 weeks. There were no differences in body mass observed between the groups. Data is expressed as mean \pm SE (n = 5).

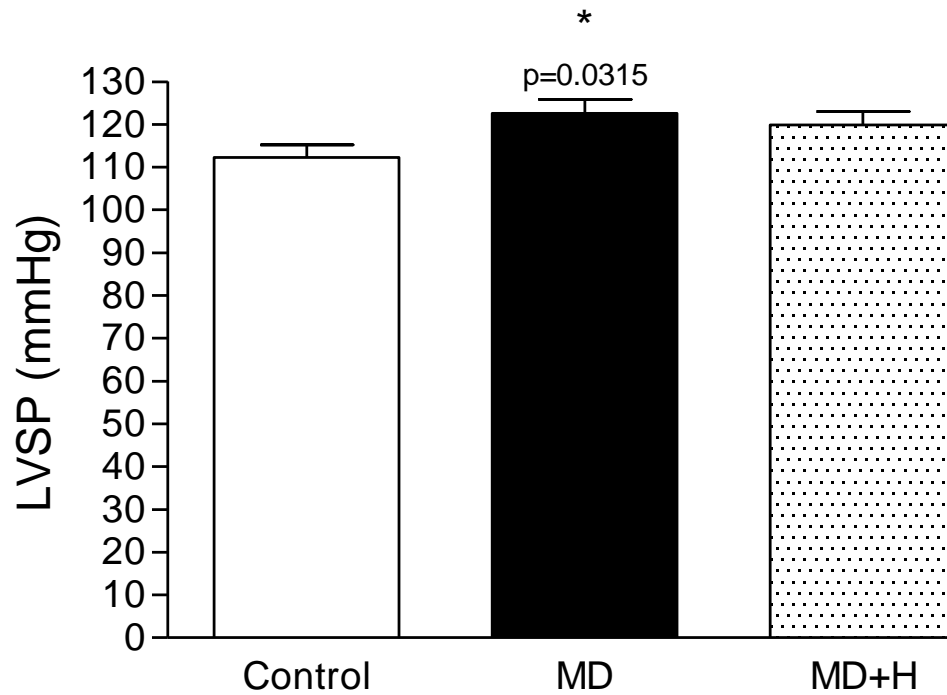


Figure 18 Effect of oral heparin (H) (1 mg/kg/48hours), given by gavage, on left ventricular systolic pressure (LVSP) in male WKY rats fed a 2% methionine diet (MD) for 8 weeks. LVSP was significantly elevated in the MD group compared to controls. Data is expressed as mean \pm SE (n = 5). *: p=0.0315 vs. control.

3.4.3. Left Ventricular End-Diastolic Pressure (LVEDP)

The effect of oral heparin on LVEDP of MD fed WKY rats including age-matched controls is shown in Figure 19. LVEDP was significantly elevated in the MD group (14.23 ± 2.5 mmHg) compared to age-matched controls (7.13 ± 1.11 mmHg, $p=0.0214$). There was no significant difference observed in LVEDP between the oral heparin group (8.42 ± 1.91 mmHg) and all other groups, suggesting oral heparin treatment attenuated the increase seen in LVEDP by MD feeding.

3.4.4. Left Ventricular Developed Pressure (LVDP)

The effect of oral heparin on LVSP of MD fed WKY rats including age-matched controls on LVDP is shown in Figure 20. In the 8 week heparin treated group exposed to a MD, LVDP was significantly elevated (111.4 ± 1.2 mmHg) as compared to its age-matched control (105.2 ± 2.2 mmHg, $p=0.0284$). There was no change observed in LVDP in the 8 week MD group (108.4 ± 3.1 mmHg) compared to controls.

3.4.5. Myocardial Indices of Contractility (+dp/dt) and Relaxation (-dp/dt)

The effect of oral heparin treatment on + dp/dt and – dp/dt is shown in Figure 21. There was no significant change in + dp/dt or – dp/dt seen with MD exposure or heparin treatment. The +dp/dt and – dp/dt were 7898 ± 357 and 6035 ± 314 mmHg/sec respectively, in the MD exposure group, 8647 ± 314 and 6455 ± 160 mmHg/sec respectively, in the diet exposure group combined with oral heparin treatment, and 8387 ± 202 and 6339 ± 117 mmHg/sec respectively in the age-matched control group.

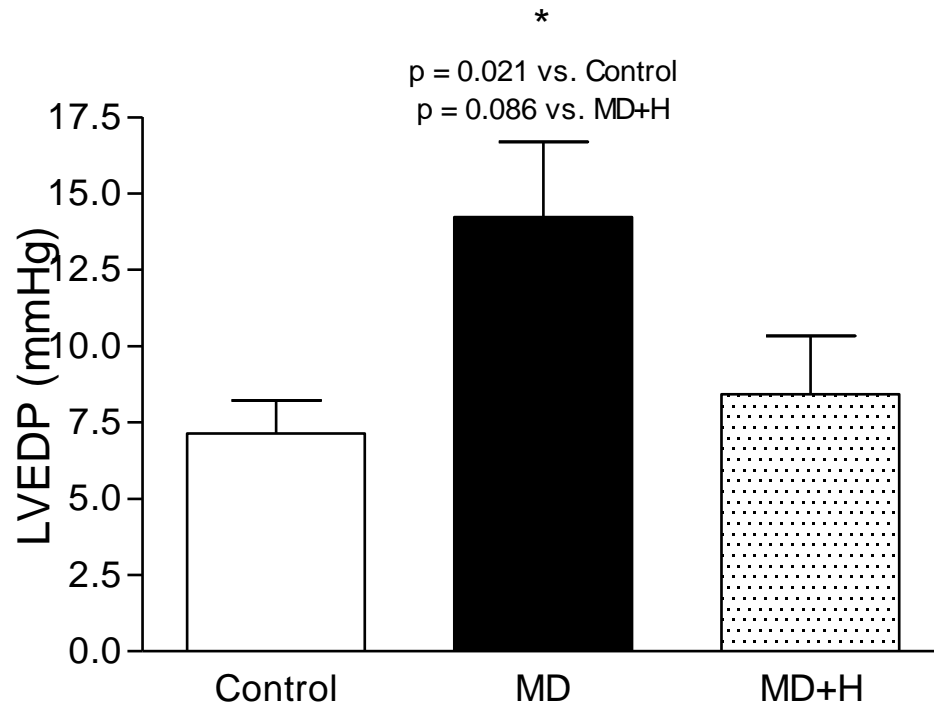


Figure 19 Effect of oral heparin (H) (1 mg/kg/48hours), given by gavage, on left ventricular end-diastolic pressure (LVEDP) measured in male WKY rats fed a 2% methionine diet (MD) for 8 weeks. LVEDP was significantly elevated in the MD group as compared to controls. No increase was seen in LVEDP in the heparin treated MD group, which was similar to control values. Data is expressed as mean \pm SE (n = 5). *: p = 0.0214 vs. control.

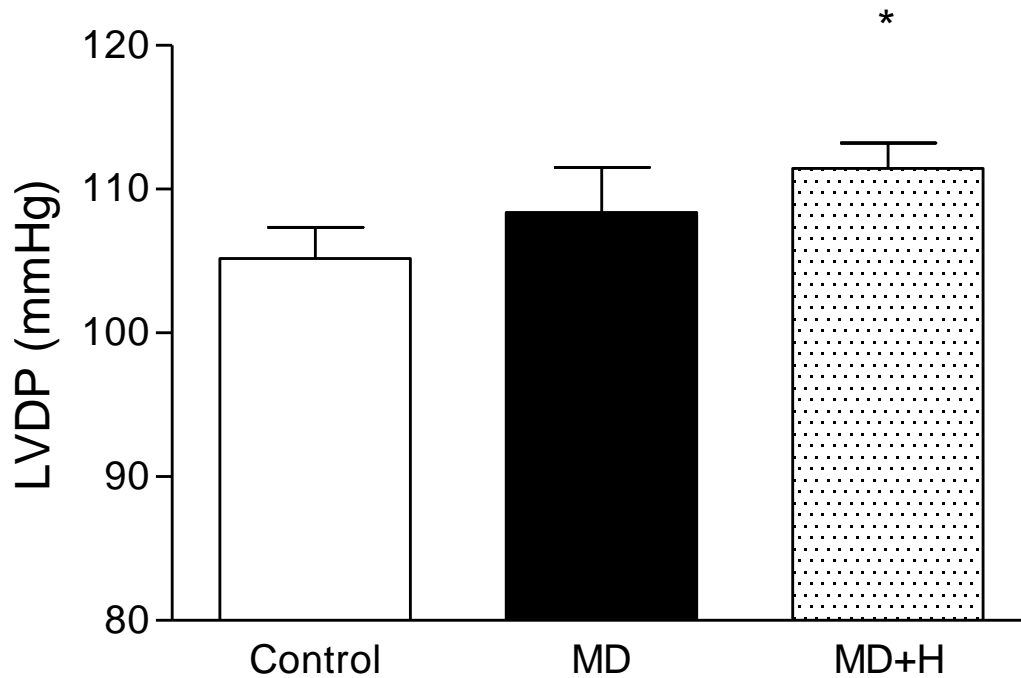


Figure 20 Effect of oral heparin (H) (1 mg/kg/48hours), given by gavage, on left ventricular developed pressure (LVDP) measured in male WKY rats fed a 2% methionine diet (MD) for 8 weeks. LVDP was significantly elevated in the MD plus oral heparin group compared to controls. There was no significant change in LVDP in the MD fed rats compared to other groups. Data is expressed as mean \pm SE (n = 5). *: p<0.05 vs. control.

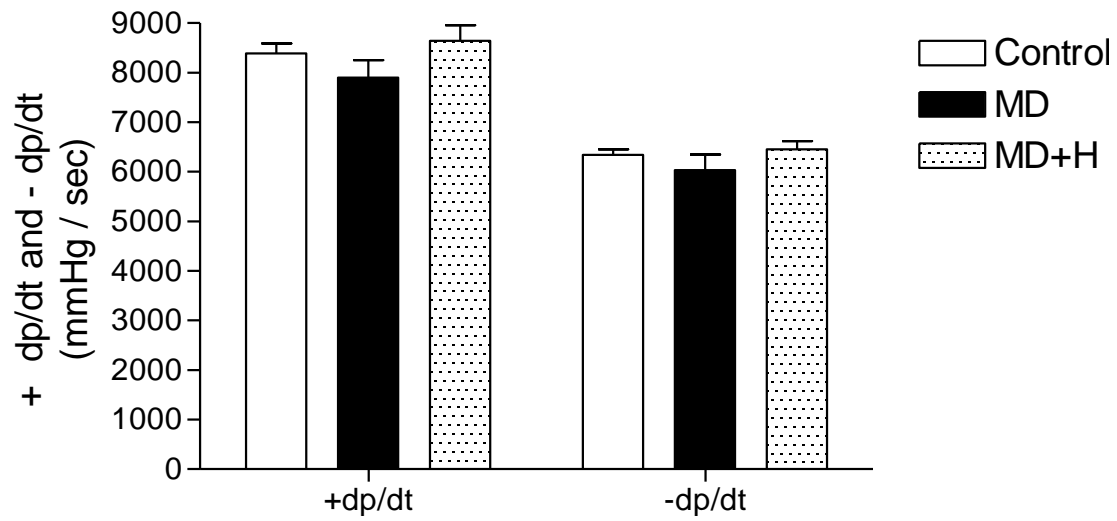


Figure 21 Effect of oral heparin (1 mg/kg/48hours), given by gavage, on myocardial indices of contractility (+ dp/dt) and relaxation (- dp/dt) measured in male WKY rats fed a 2% methionine diet (MD) for 8 weeks. No significant difference was observed between any of the groups. Data is expressed as mean \pm SE (n = 5).

3.4.6. Left Ventricular Mass and Volume

The mass and volume of the LV was measured following the hemodynamic investigations, results are shown in Figure 22. There were no differences observed in the LV mass (control: 0.74 ± 0.07 g; MD: 0.69 ± 0.02 g; MD+H: 0.73 ± 0.006 g) or volume (control: 0.58 ± 0.12 mL; MD: 0.85 ± 0.10 mL; MD+H: 0.83 ± 0.04 mL) between any of the groups. There was a trend towards increased LV volume in the MD fed rats ($p=0.086$, control vs. MD).

3.4.7. Arterial Pressures

The effect of oral heparin treatment on SP, DP and MAP of MD fed WKY rats is shown in Figure 23. All measures of arterial pressure, SP, DP and MAP were significantly elevated in the MD exposed group (119.9 ± 3.92 mmHg; 90.33 ± 3.51 mmHg; 97.67 ± 2.91 mmHg, respectively) compared to age-matched controls (107.8 ± 2.507 mmHg, $p=0.020$; 79.24 ± 2.081 mmHg, $p=0.017$; 88.77 ± 2.182 mmHg, $p=0.028$; respectively). There was no difference in arterial pressures between the MD plus oral heparin group (SP: 114.7 ± 3.3 mmHg; DP: 83.4 ± 2.4 mmHg; MAP: 93.82 ± 2.7 mmHg) vs. controls or the MD alone group.

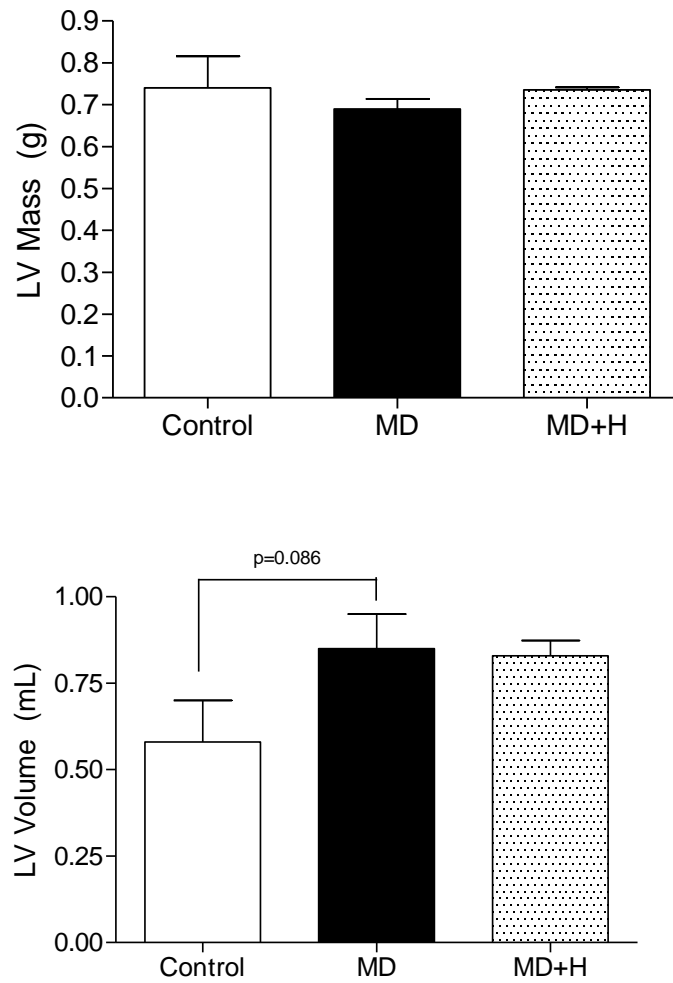


Figure 22 Effect of oral heparin (H) (1 mg/kg/48hours), given by gavage, on LV mass and volume measured in male WKY rats fed a 2% methionine diet (MD) for 8 weeks. There were no differences observed between the LV mass or volume in any of the groups. Data is expressed as mean \pm SE (n = 5). p=0.086 MD vs. C.

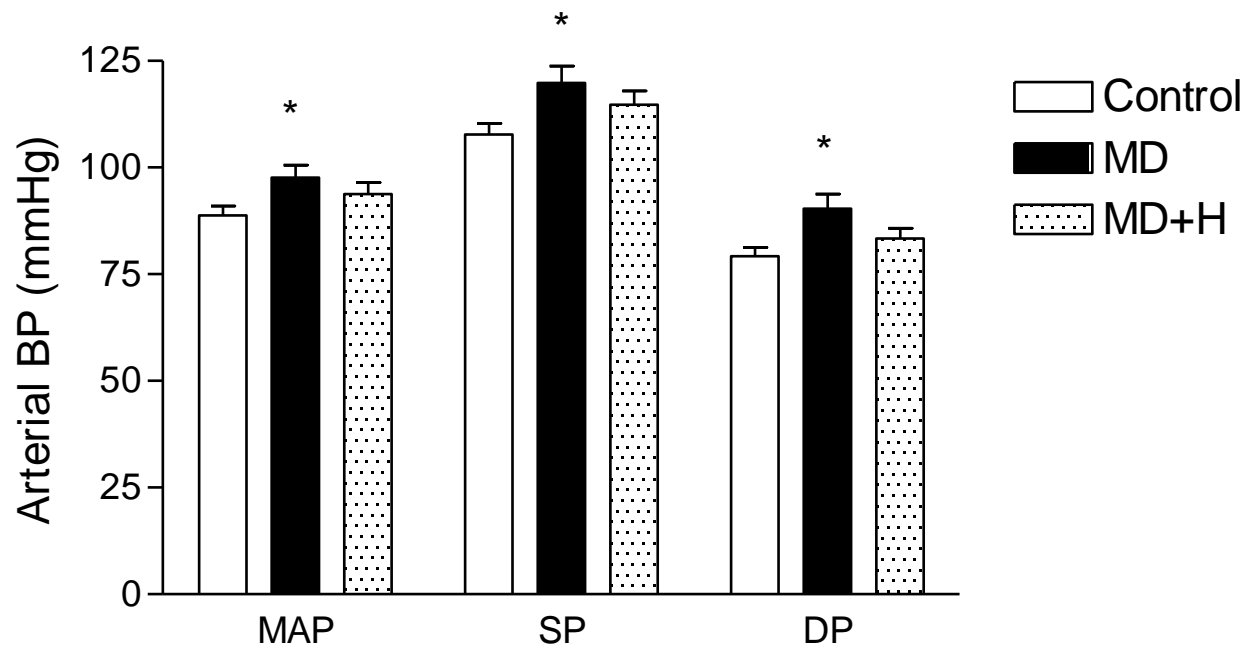


Figure 23 Effect of oral heparin (H) (1 mg/kg/48hours), given by gavage, on mean arterial pressure (MAP, systolic pressure (SP) and diastolic pressure (DP) measured in male WKY rats fed a 2% methionine diet (MD) for 8 weeks. All measures of arterial pressure are significantly elevated in the rats exposed to MD for 8 weeks compared to their age-matched control. There were no differences observed between MD plus oral heparin arterial pressures and the other groups. Data is expressed as mean \pm SE (n = 5). *: $p < 0.05$ MD vs. Control.

3.5. EFFECT OF ORAL HEPARIN TREATMENT ON MD FED WKY RATS FOR 8 WEEKS ON MALONDIALDEHYDE (MDA) CONCENTRATIONS

3.5.1. Serum MDA

The effect of oral heparin treatment in MD fed male WKY rats on serum MDA concentrations is shown in Figure 24 and Table 1. Neither MD exposure nor oral heparin treatment exposure had any significant effect on serum MDA concentrations.

3.5.2. Liver MDA

The effect of oral heparin treatment in MD fed male WKY rats on liver MDA concentrations is shown in Figure 25 and Table 1. Oral heparin treatment significantly decreased liver MDA concentrations compared to the MD group ($p=0.0317$). There was no difference in liver MDA concentrations between the control group (4.179 ± 1.1 nmol/mg protein) and other groups.

3.5.3. Kidney MDA

The effect of oral heparin treatment in MD fed male WKY rats on kidney MDA concentrations is shown in Figure 26 and Table 1. Exposure to a MD for 8 weeks produced a significant decrease in kidney MDA levels compared to the control group ($p=0.0159$). Oral heparin treatment had no significant effect on kidney MDA levels compared to other groups.

3.5.4. Mesentery MDA

The effect of oral heparin treatment in MD fed male WKY rats on mesentery MDA concentrations is shown in Figure 27 and Table 1. There was no significant difference observed in mesentery MDA concentrations between groups.

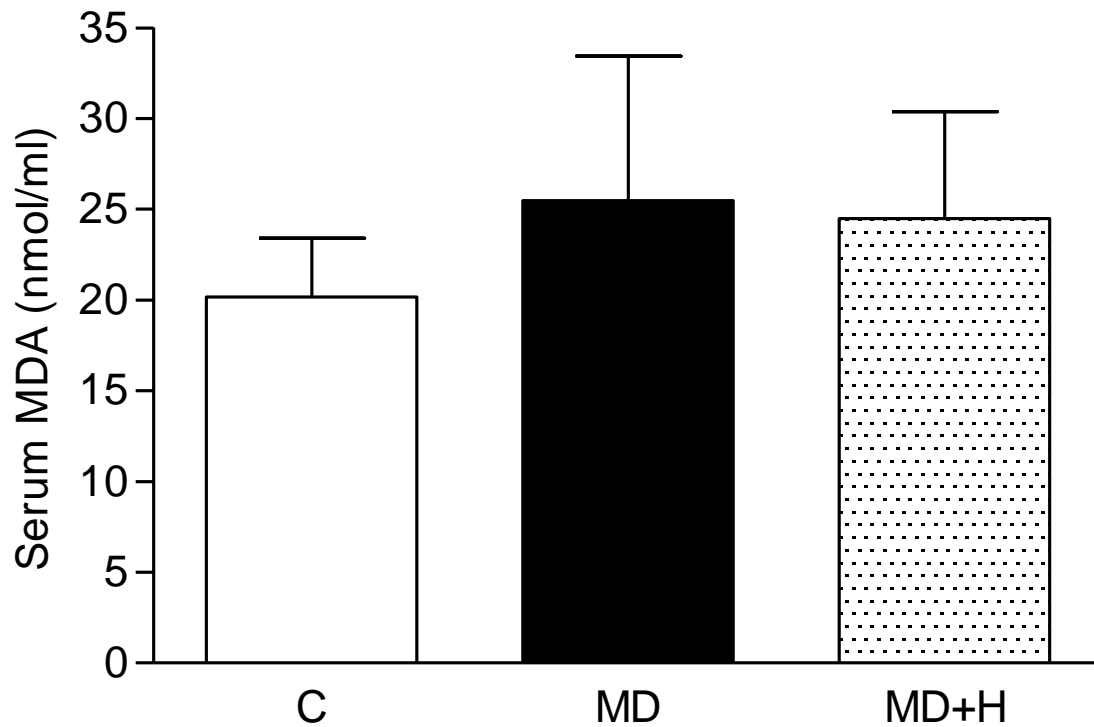


Figure 24 Effect of oral heparin (H) (1 mg/kg/48hours), given by gavage, on malondialdehyde (MDA) concentration measured in serum samples from male WKY rats fed a 2% methionine diet (MD) for 8 weeks. No significant differences were observed between groups. Data is expressed as mean \pm SE (n=5).

Table 1: Effect of oral heparin (1 mg/kg/48hours), given by gavage, on malondialdehyde (MDA) concentration measured in serum, liver, kidney, first-order mesentery, descending thoracic aortic and left ventricle samples from male WKY rats fed a high methionine diet (MD) for 8 weeks.

	[MDA] nmol/ml	[MDA] nmol/mg protein				
	Serum	Liver	Kidney	Mesentery	Aorta	Left Ventricle
Control	20.16 ± 3.2	4.179 ± 1.1	3.263 ± 0.66	1.869 ± 0.71	3.801 ± 1.7	0.7720 ± 0.22
MD	25.47 ± 8.0	5.100 ± 0.96	1.586 ± 0.12†	0.5654 ± 0.10	1.990 ± 0.68	0.2810 ± 0.06
MD+H	24.49 ± 5.9	2.423 ± 0.28*	2.287 ± 0.73	0.5910 ± 0.15	2.420 ± 0.44	0.5930 ± 0.14

MD: Methionine Diet; MD+H Methionine Diet + Oral Heparin Treatment. Data is expressed as mean ± SEM. * p=0.0317 vs. MD. † p=0.0159 vs. Control.

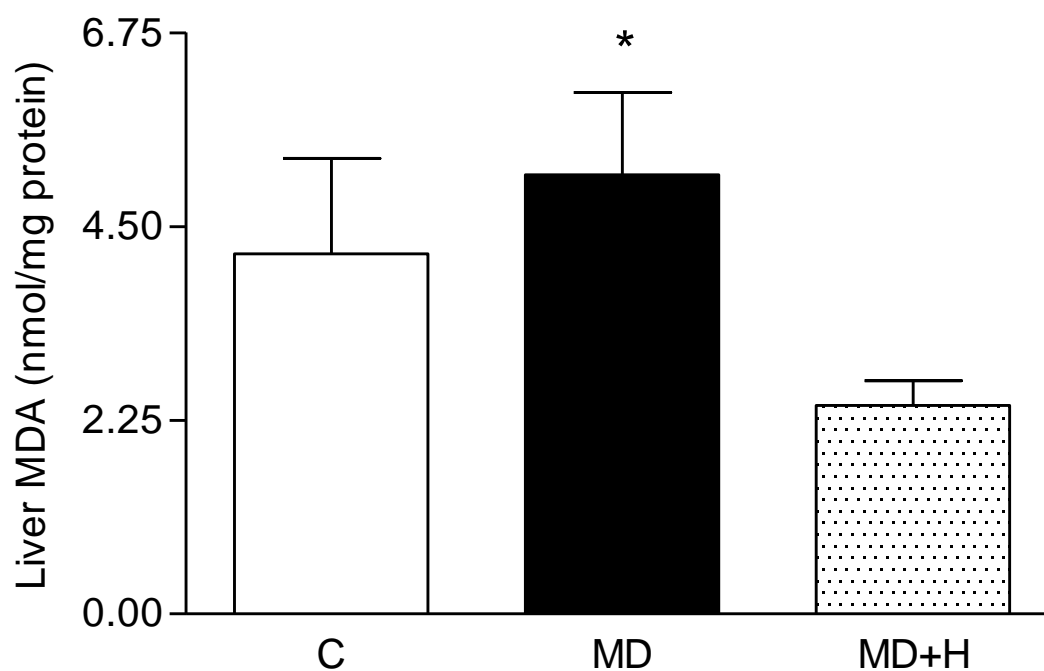


Figure 25 Effect of oral heparin (H) (1 mg/kg/48hours), given by gavage, on malondialdehyde (MDA) concentration measured in liver samples from male WKY rats fed a 2% methionine diet (MD) for 8 weeks. The rats exposed to MD had significantly higher MDA levels than diet exposed rats treated with oral heparin. Data is expressed as mean \pm SE (n=5). *: p=0.0317 vs. MD+H.

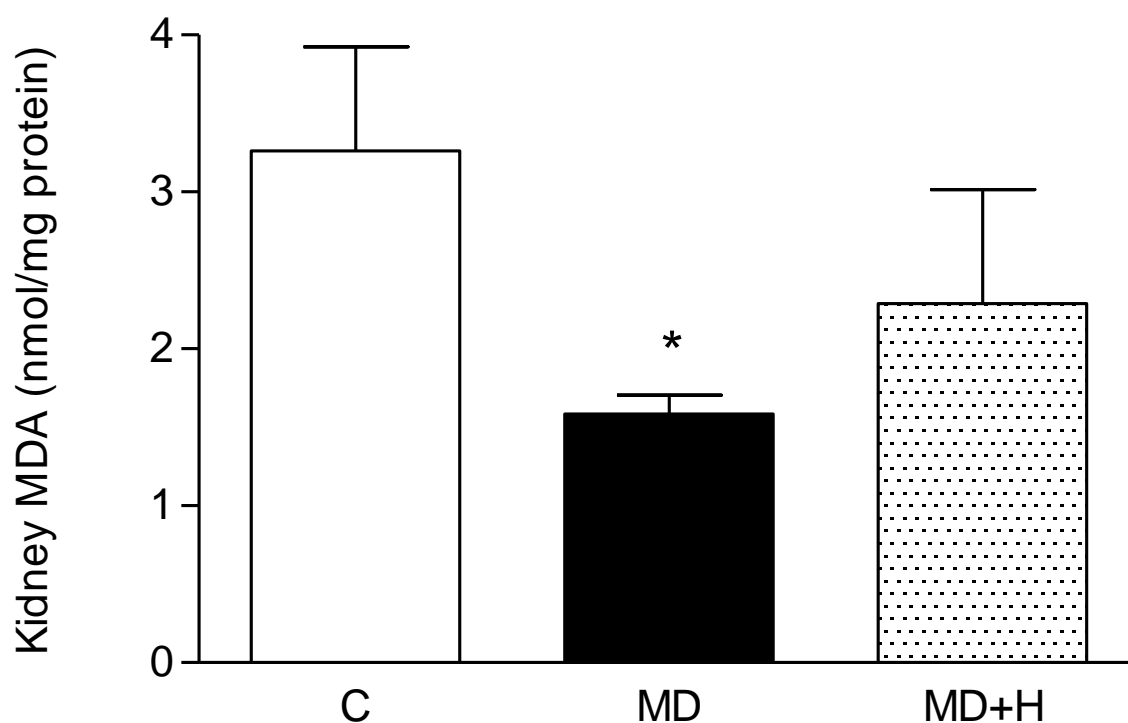


Figure 26 Effect of oral heparin (H) (1 mg/kg/48hours), given by gavage, on malondialdehyde (MDA) concentration measured in kidney samples from male WKY rats fed a 2% methionine diet (MD) for 8 weeks. Rats exposed to a MD for 8 weeks had significantly lower MDA levels compared to controls. Data is expressed as mean \pm SE (n=5). *: p=0.0179 vs. control.

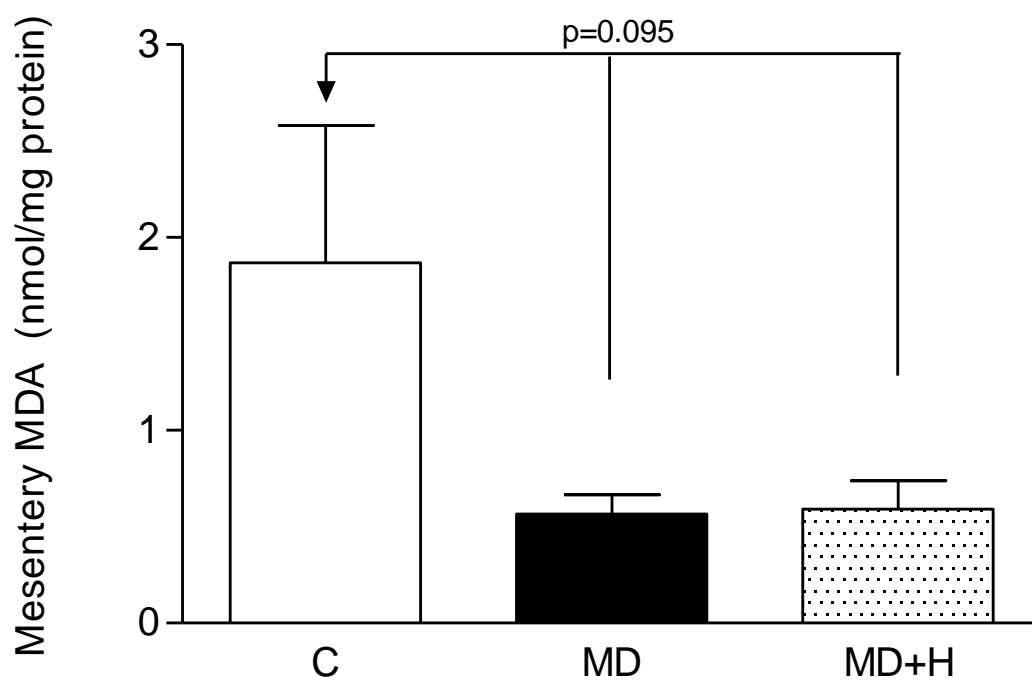


Figure 27 Effect of oral heparin (H) (1 mg/kg/48hours), given by gavage, on malondialdehyde (MDA) concentration measured in first-order mesentery samples from male WKY rats fed a 2% methionine diet (MD) for 8 weeks. No significant differences were observed between groups. Data is expressed as mean \pm SE (n=5).

3.5.5. Aorta MDA

The effect of oral heparin treatment in MD fed male WKY rats on aorta MDA concentrations is shown in Figure 28 and Table 1. There was a trend towards a decrease in MDA concentrations in the MD group with and without heparin treatment. However, this difference compared to control failed to reach significance ($p=0.095$ for MD and $p=0.31$ for MD+H).

3.5.6. Left ventricle MDA

The effect of oral heparin treatment in MD fed male WKY rats on LV MDA concentrations is shown in Figure 29 and Table 1. There were no significant differences observed in LV MDA between all groups. There was a trend towards a decrease in LV MDA concentrations in the MD exposure without heparin treatment compared to control ($p=0.095$). Oral heparin appeared to reverse the effect of MD on MDA levels, by returning values close to control.

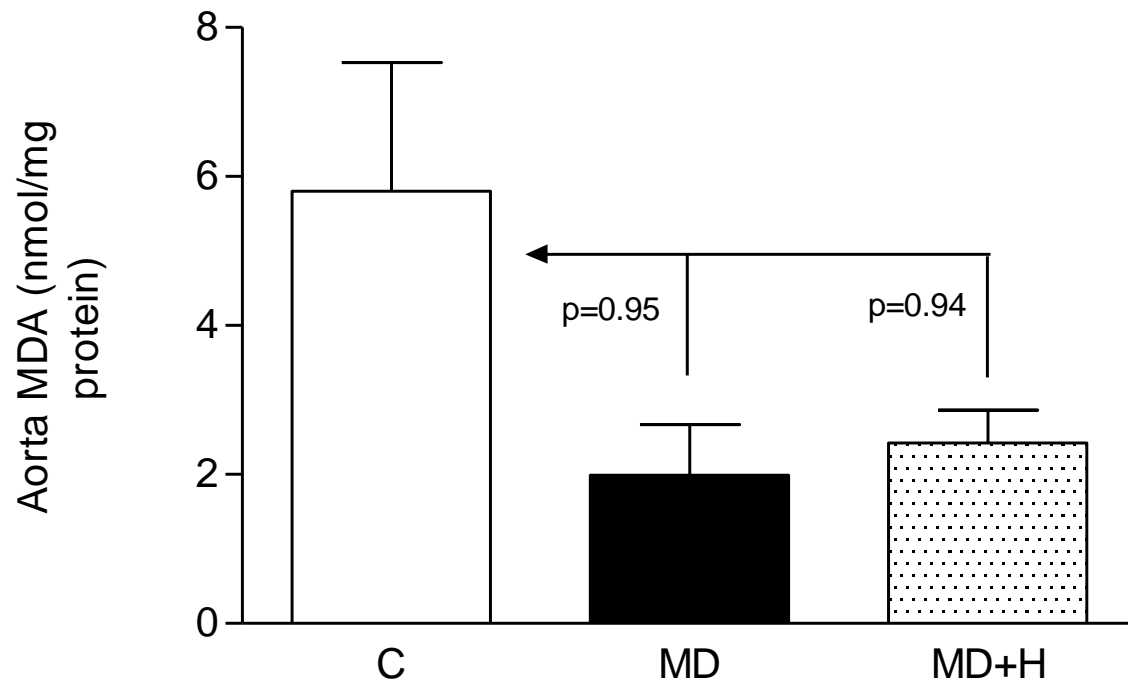


Figure 28 Effect of oral heparin (H) (1 mg/kg/48hours), given by gavage, on malondialdehyde (MDA) concentration measured in descending thoracic aortic samples from male WKY rats fed a 2% methionine diet (MD) for 8 weeks. No significant differences were observed between groups. Data is expressed as mean \pm SE (n=5).

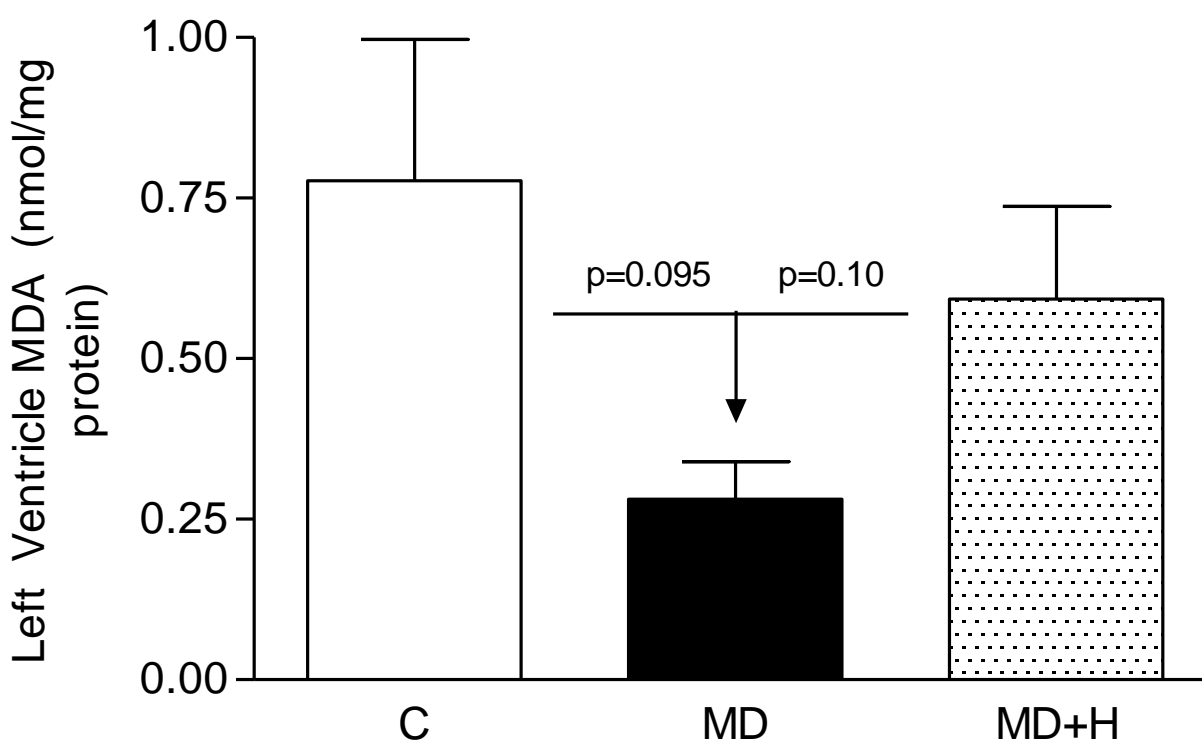


Figure 29 Effect of oral heparin (H) (1 mg/kg/48hours), given by gavage, on malondialdehyde (MDA) concentration measured in Left Ventricle samples from male WKY rats fed a 2% methionine diet (MD) for 8 weeks. No significant differences were observed between groups. Data is expressed as mean \pm SE (n=5).

3.6. EFFECT OF ORAL HEPARIN TREATMENT ON MD FED WKY RATS FOR 8 WEEKS ON GLUTATHIONE CONCENTRATIONS

Total reduced fraction of glutathione (GSH) measured by HPLC to determine the effects of oral heparin (1mg/kg/48hr) given by gavage, in male WKY rats fed a 2% methionine diet (MD) for 8 weeks. GSH was measured in plasma samples as well as liver, kidney and left ventricular tissue samples for each group.

3.6.1. Plasma GSH

The effect of oral heparin (1mg/kg/48hr) given by gavage, in male WKY rats fed a 2% methionine diet (MD), on total plasma GSH concentrations is shown in Figure 30 and Table 2. The plasma GSH concentrations in the MD group were significantly lower than the control group ($p=0.016$). MD+H plasma GSH concentrations did not differ from control and MD groups ($p=0.16$ and $p=0.41$ respectively).

3.6.2. Liver GSH

The effect of oral heparin (1mg/kg/48hr) given by gavage, in male WKY rats fed a 2% methionine diet (MD), on liver GSH concentrations is shown in Figure 31 and Table 2. Liver GSH concentrations were not significantly different between groups (control vs. MD: $p=0.62$; control vs. MD+H: $p=0.70$; MD vs. MD+H: $p=0.87$).

Table 2: The effect of oral heparin treatment (H) (1/mg/kg/48hrs) given by gavage, in male WKY rats fed a high methionine diet (MD) for 8 weeks on reduced glutathione (GSH) concentrations in plasma, liver, kidney, and LV samples.

	[GSH] $\mu\text{mol/l}$	[GSH] $\mu\text{mol/mg protein}$		
	Plasma	Liver	Kidney	Left Ventricle
Control	4.239 ± 0.94	0.02786 ± 0.0029	0.01690 ± 0.0016	0.02074 ± 0.0050
MD	$0.5896 \pm 0.59^{\dagger}$	0.02460 ± 0.0025	0.01830 ± 0.0025	0.01353 ± 0.0016
MD+H	1.817 ± 1.2	0.02880 ± 0.0036	0.03438 ± 0.0073	$0.02288 \pm 0.0023^*$

MD: Methionine Diet; MD+H: Methionine Diet + Oral Heparin Treatment. Data is expressed as mean \pm SEM. (n = 5). * p=0.0150 vs. MD. \dagger p=0.016 vs. control.

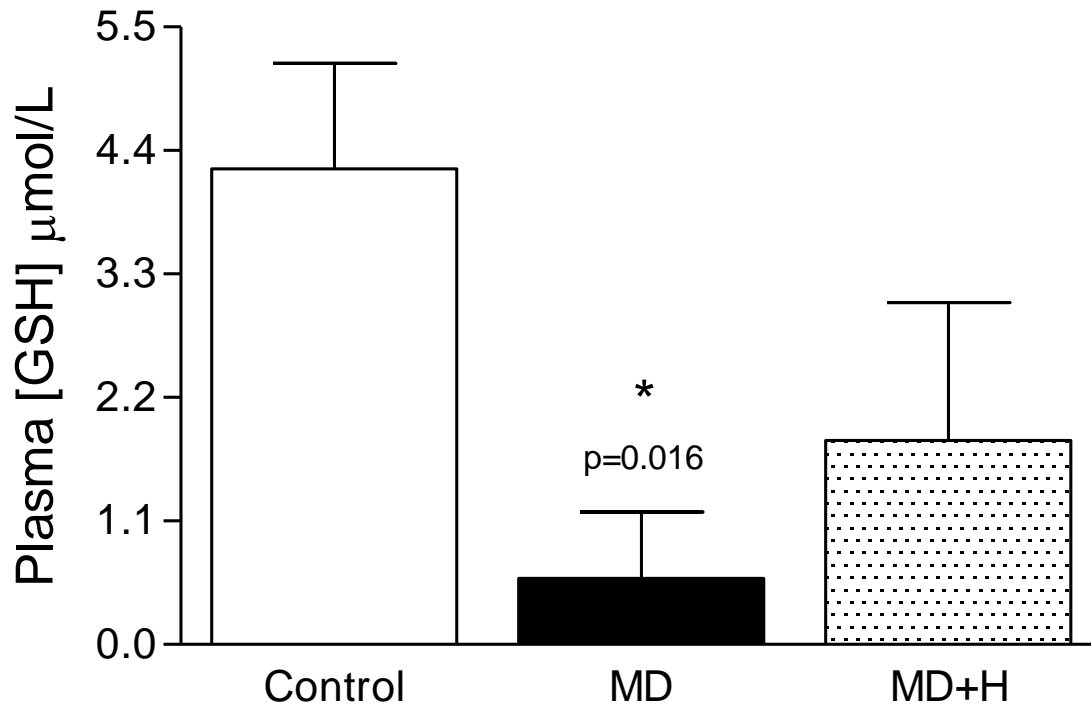


Figure 30 Effect of oral heparin (H) (1 mg/kg/48hours), given by gavage, on GSH concentrations in plasma samples from male WKY rats fed a 2% methionine diet (MD) for 8 weeks. Plasma GSH concentration in the MD group were significantly lower than the control group ($p=0.016$ vs. control). Data is expressed as mean \pm SE ($n = 5$).

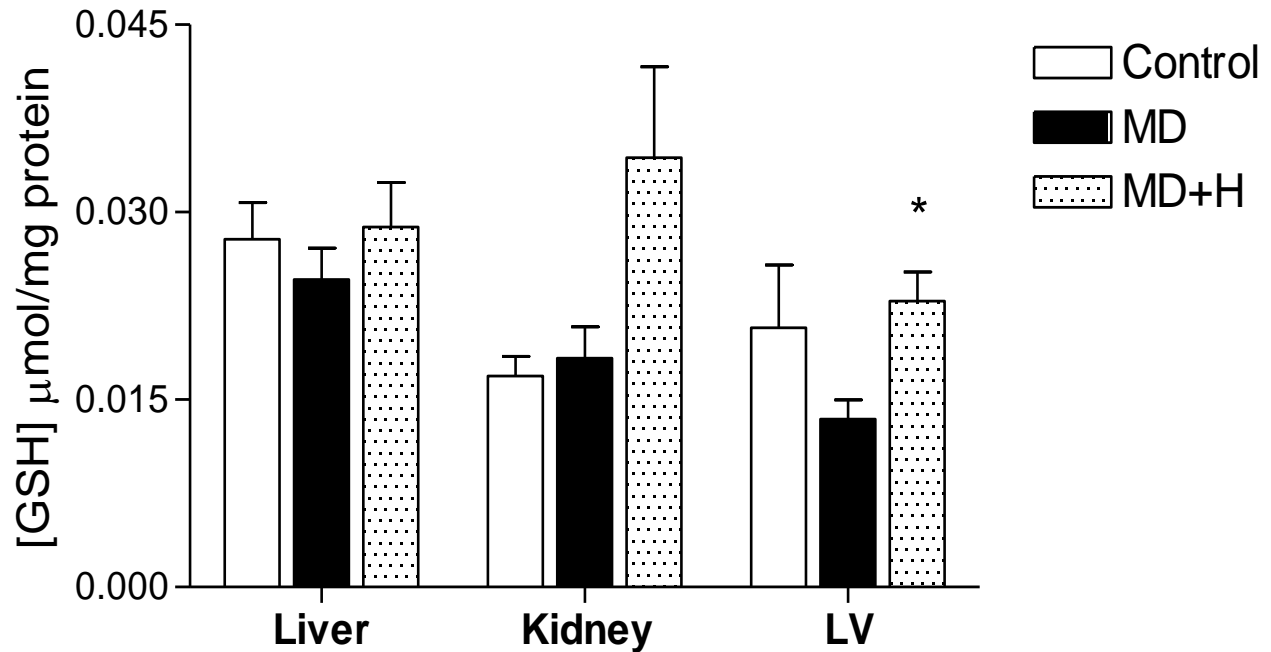


Figure 31 Effect of oral heparin (H) (1 mg/kg/48hours), given by gavage, on GSH concentrations in liver, kidney and left ventricle tissue samples from male WKY rats fed a 2% methionine diet (MD) for 8 weeks. There was no significant difference in GSH concentrations between groups in either the liver or kidney tissue analysis. However, the oral heparin treated MD group had significantly higher LV GSH concentrations compared to MD only group. Data is expressed \pm SE (n = 5). * p=0.0150.

3.6.3. Kidney GSH

The effect of oral heparin (1mg/kg/48hr) given by gavage, in male WKY rats fed a 2% methionine diet (MD), on kidney GSH concentrations is shown in Figure 31 and Table 2. There was a trend of increasing kidney GSH concentrations in the oral heparin treated MD group, which failed to reach significance when compared to the control and MD group ($p=0.078$; $p=0.10$, respectively). Difference in kidney GSH concentrations were also non-significant when the MD and MD+H groups were compared ($p=0.65$).

3.6.4. Left ventricle GSH

The effect of oral heparin (1mg/kg/48hr) given by gavage, in male WKY rats fed a 2% methionine diet (MD), on left ventricle GSH concentrations is shown in Figure 31 and Table 2. The oral heparin treated group had significantly higher LV GSH values than the MD group ($p=0.015$). The LV GSH concentrations in the control group were not significantly different compared to the other groups (vs. MD: $p=0.14$; vs. MD+H: $p=0.86$).

3.7. EFFECT OF ORAL HEPARIN TREATMENT OF MD FED WKY RATS FOR 8 WEEKS ON ENDOTHELIAL CELL APOPTOSIS MEASURED BY TUNEL STAIN

The percent of apoptotic endothelial cells (ECs) as measured by fluorescent TUNEL stain was measured to determine the effect of oral heparin (1mg/kg/48hr) given by gavage, in male WKY rats fed a 2% methionine diet (MD) for 8 weeks. Apoptotic ECs were measured in aorta, renal artery and superior mesenteric artery samples for each group.

3.7.1. Apoptotic Aortic ECs

The effect of oral heparin (1mg/kg/48hr) given by gavage, in male WKY rats fed a 2% methionine diet (MD) for 8 weeks, on EC apoptosis in the aorta is shown in Figures 32 and 35 and representative TUNEL stains shown in Figures 36 – 38. The percent of apoptotic aortic ECs were significantly higher in the MD group ($17.04 \pm 3.74\%$) compared to controls ($6.08 \pm 3.24\%$, $p=0.036$). The heparin treatment group percent aortic apoptotic ECs were not significantly different from either group ($13.38 \pm 4.38\%$). Interestingly, there was a trend towards a higher percent apoptotic ECs in the aorta in the MD group compared to the renal artery ($p=0.075$).

3.7.2. Apoptotic Renal Artery ECs

The effect of oral heparin (1mg/kg/48hr) given by gavage, in male WKY rats fed a 2% methionine diet (MD) for 8 weeks, on EC apoptosis in the renal artery is shown in Figures 33 and 35 and representative TUNEL stains shown in Figures 39 – 41. There was no significant difference in the percent of apoptotic renal artery ECs between the groups (Control: $5.86 \pm 2.15\%$; MD: $7.81 \pm 3.25\%$; MD+H: $10.59 \pm 3.55\%$).

3.7.3. Apoptotic Mesenteric Artery ECs

The effect of oral heparin (1mg/kg/48hr) given by gavage, in male WKY rats fed a 2% methionine diet (MD) for 8 weeks, on EC apoptosis in the superior mesenteric artery is shown in Figures 34 and 35 and representative TUNEL stains shown in Figures 42 – 44. The percent of apoptotic EC in the mesenteric artery was significantly elevated in the MD group ($17.99 \pm 1.90\%$) as compared with controls ($7.43 \pm 1.62\%$, $p=0.0003$). Heparin treatment significantly reduced the percent apoptotic EC in MD treated mesenteric artery ($7.31 \pm 1.17\%$, $p=0.0001$) to values very similar to control ($p=0.95$). Interestingly, the percent apoptotic ECs in the MD group mesenteric artery were significantly higher than the MD renal artery ($p=0.014$).

Table 3: The total number and % of apoptotic endothelial cells counted in aorta, superior mesenteric artery and renal artery tissue samples of male WKY rats fed a high methionine diet (MD) for 8 weeks and treated with oral heparin (H) (1/mg/kg/48hrs) given by gavage.

	Aorta		Mesenteric Artery		Renal Artery	
	Total # of cells counted	% Apoptotic	Total # of cells counted	% Apoptotic	Total # of cells counted	% Apoptotic
Control	100	6.08 ± 3.24	397	7.43 ± 1.62	389	5.86 ± 2.15
MD	161	17.04 ± 3.74	344	17.99 ± 1.90	359	7.81 ± 3.25
MD+H	69	13.38 ± 4.38	446	7.31 ± 1.17	327	10.59 ± 3.55

MD: Methionine Diet; MD+H: Methionine Diet + Oral Heparin Treatment. Data is expressed as mean ± SEM. (n = 10 – 15).

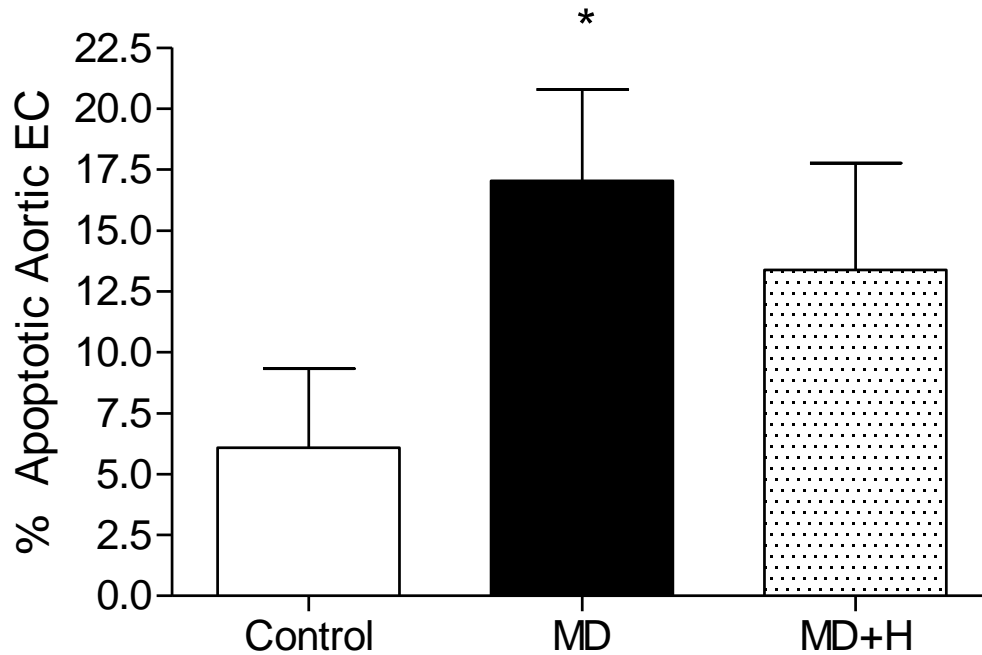


Figure 32 Percent apoptotic aortic ECs from male WKY rats fed MD for 8 weeks, including age-matched controls and oral heparin treated (H) (1 mg/kg/48hours) MD rats (MD+H). Percent of apoptotic cells were significantly higher in the MD group ($17.04 \pm 3.74\%$) compared to controls ($6.08 \pm 3.24\%$). There was no difference between MD+H values and the other groups. Data is expressed as mean \pm SE (n=15). *: $p=0.036$ vs. control.

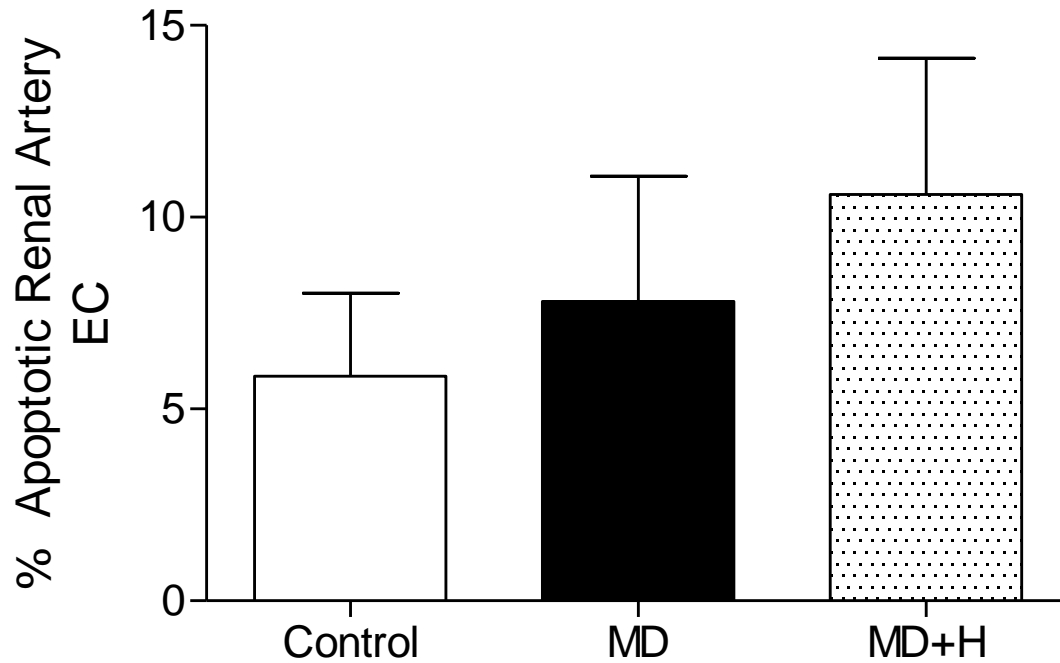


Figure 33 Percent apoptotic renal artery ECs from male WKY rats fed MD for 8 weeks, including age-matched controls and oral heparin treated (H) (1 mg/kg/48hours) MD rats (MD+H). There were no significant differences between the groups. Data is expressed as mean \pm SE (n=15).

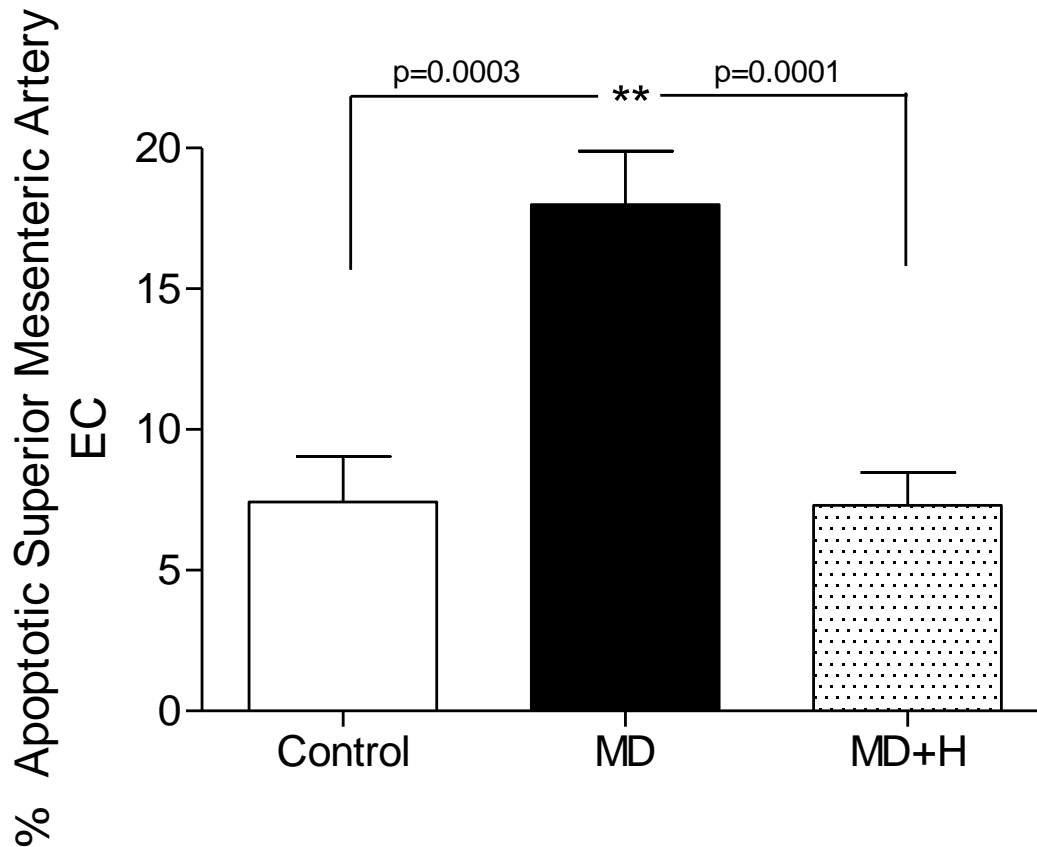


Figure 34 Percent apoptotic mesenteric artery ECs from male WKY rats fed MD for 8 weeks, including age-matched controls and oral heparin treated (H) (1 mg/kg/48hours) MD rats (MD+H). The percent of apoptotic ECs were significantly higher in the MD treated group ($17.99 \pm 1.90\%$) compared to controls and the heparin treated group (7.43 ± 1.62 , $p=0.0003$; $7.31 \pm 1.18\%$, $p=0.0001$, respectively). Data is expressed as mean \pm SE ($n=15$). **: $p<0.001$ vs. control and MD+H.

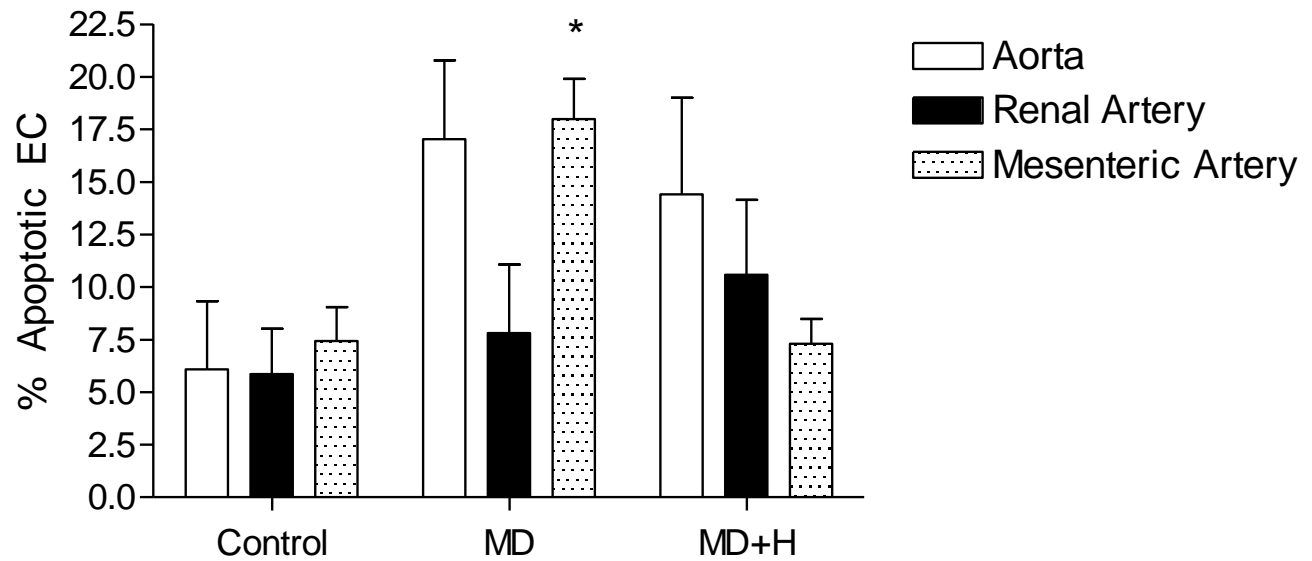


Figure 35 Percent apoptotic ECs comparison between the male WKY rats fed MD for 8 weeks, controls and oral heparin treated (1 mg/kg/48hours) MD rats (MD+H). The percent of apoptotic ECs were significantly higher in the mesenteric artery of the MD treated group ($17.99 \pm 1.90\%$) compared to the renal artery in the MD group ($7.82 \pm 3.25\%$, $p=0.014$). There was a trend for that the aorta had a higher percentage of apoptotic ECs in the MD group ($17.04 \pm 3.74\%$) compared to the renal artery, yet this difference is non-significant ($p=0.075$). Data is expressed as mean \pm SE (n=15). *: $p=0.014$ vs. Renal Artery.

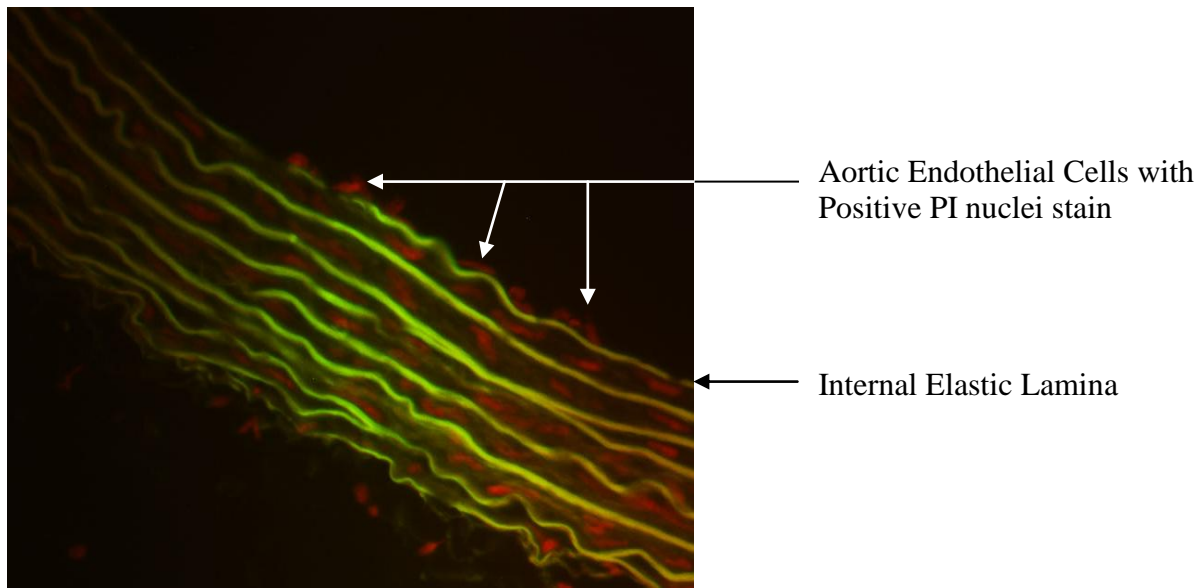


Figure 36 Control Aorta; 20x magnification

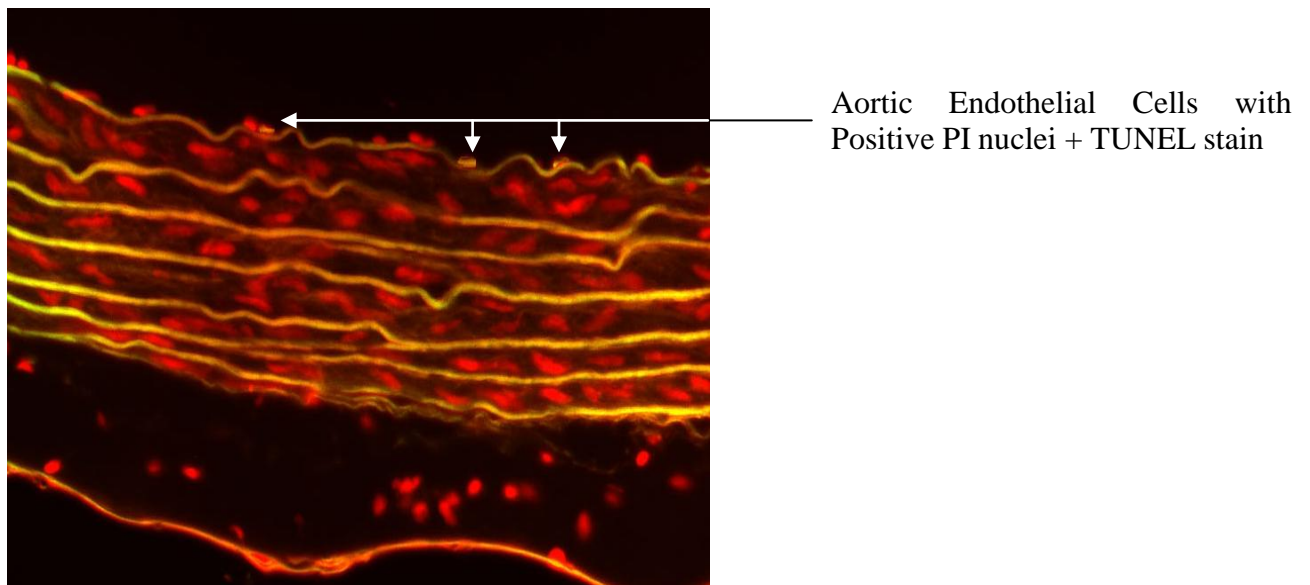


Figure 37 MD Aorta; 20x magnification

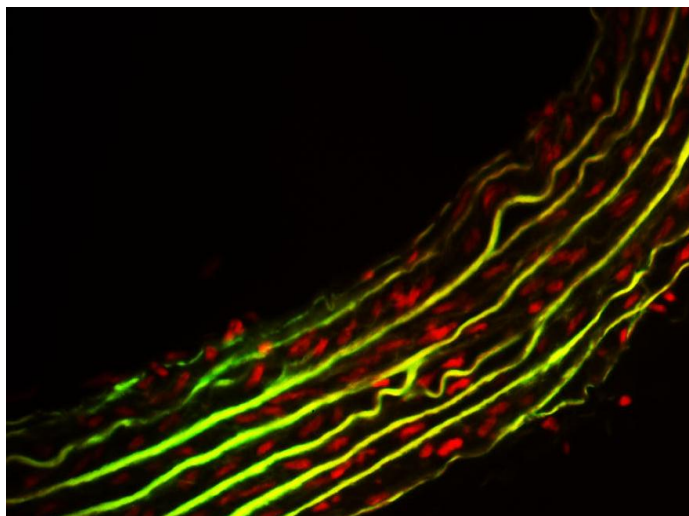


Figure 38 MD+H Aorta; 20x magnification

Figures 35 – 38 Representative TUNEL double-stained images of aortas dissected from male WKY rats fed MD for 8 weeks, controls and oral heparin treated (1 mg/kg/48hours) MD rats (MD+H). Images for TUNEL staining have an overlay of green fluorescence from the TUNEL stain with red fluorescence from PI. Image is at 20x magnification. Endothelial cells were counted as all cells above the internal elastic lamina with a positive PI nuclei stain. TUNEL-positive endothelial cells were counted as all cells in the overlaid image, above the internal elastic lamina which appear yellow. Percent apoptotic aortic endothelial cells were quantified in ratio as number of TUNEL-positive endothelial cells: number of endothelial cells.

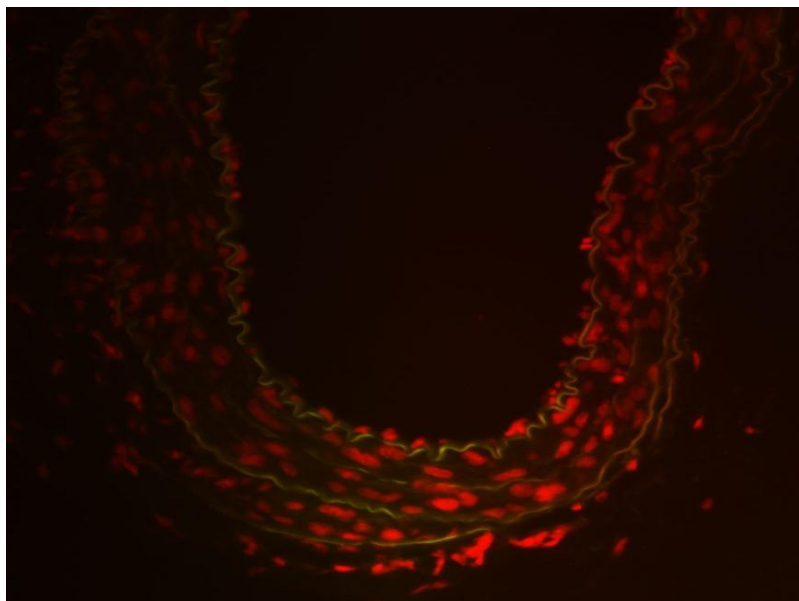


Figure 39 Control Renal Artery; 20x magnification

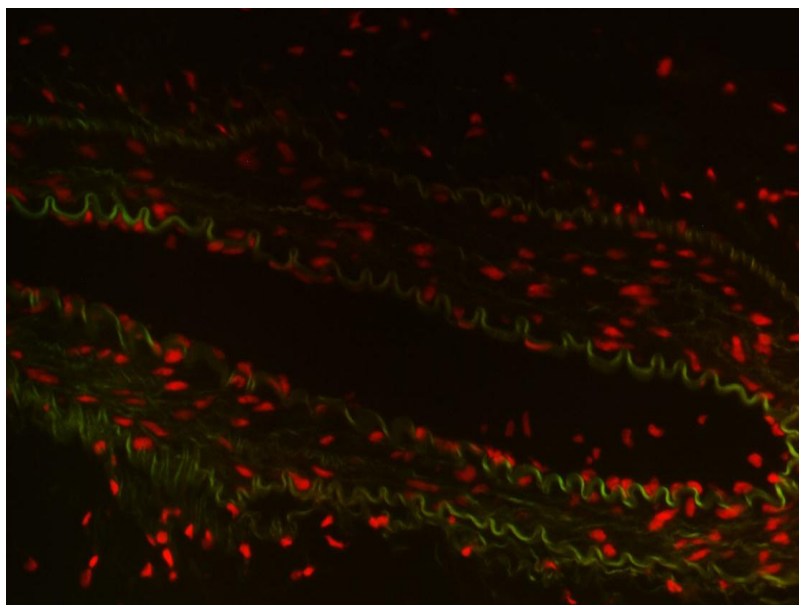


Figure 40 MD Renal Artery; 20x magnification

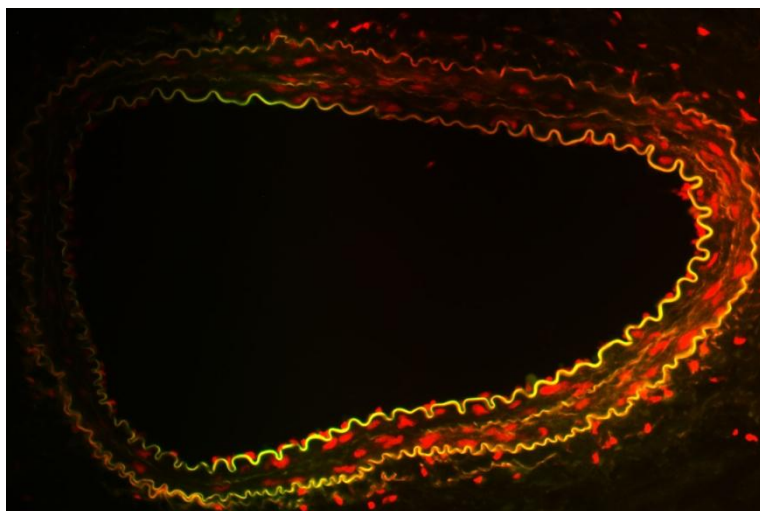


Figure 41 MD+H Renal Artery; 20x magnification

Figures 39 – 41 Representative TUNEL double-stained images of renal arteries dissected from male WKY rats fed MD for 8 weeks, controls and oral heparin treated (1 mg/kg/48hours) MD rats (MD+H). Images for TUNEL staining have an overlay of green fluorescence from the TUNEL stain with red fluorescence from PI. Image is at 20x magnification. Endothelial cells were counted as all cells above the internal elastic lamina with a positive PI nuclei stain. TUNEL-positive endothelial cells were counted as all cells in the overlaid image, above the internal elastic lamina which appear yellow. Percent apoptotic aortic endothelial cells were quantified in ratio as number of TUNEL-positive endothelial cells: number of endothelial cells.

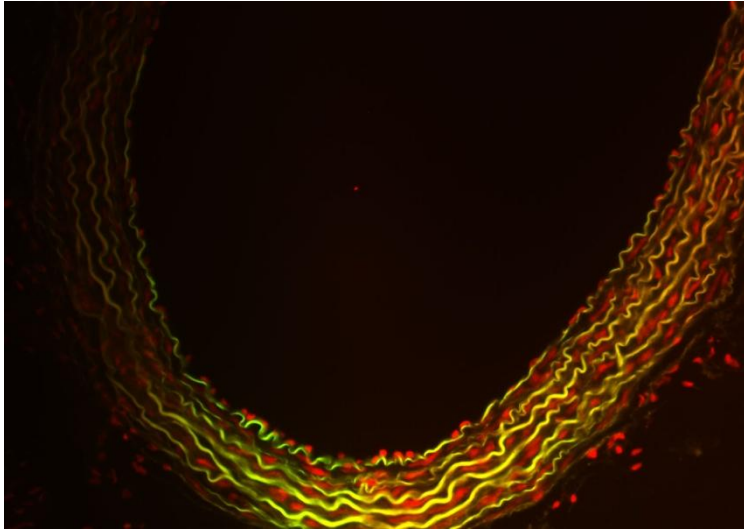


Figure 42 Control Superior Mesenteric Artery; 20x magnification

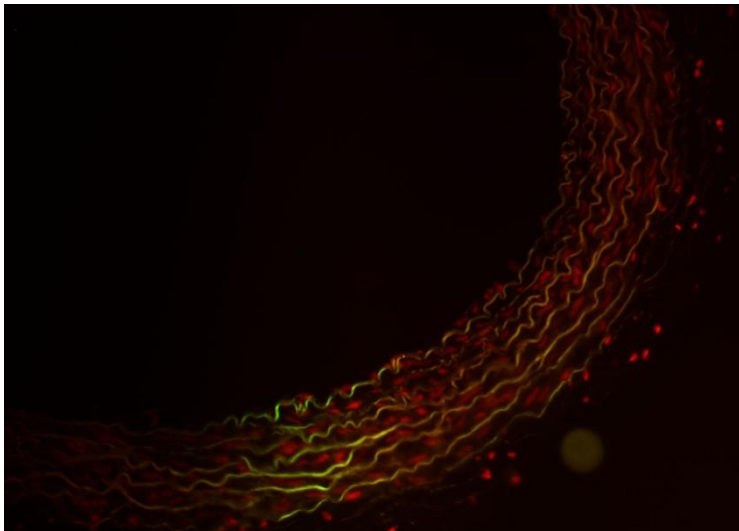


Figure 43 MD Superior Mesenteric Artery; 20x magnification

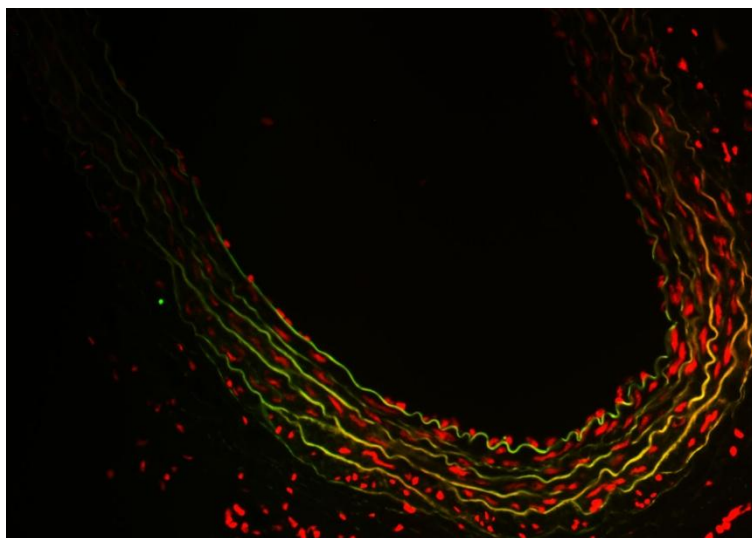


Figure 44 MD+H Superior Mesenteric Artery; 20x magnification

Figures 42 – 44 Representative TUNEL double-stained images of the superior mesenteric arteries dissected from male WKY rats fed MD for 8 weeks, controls and oral heparin treated (1 mg/kg/48hours) MD rats (MD+H). Images for TUNEL staining have an overlay of green fluorescence from the TUNEL stain with red fluorescence from PI. Image is at 20x magnification. Endothelial cells were counted as all cells above the internal elastic lamina with a positive PI nuclei stain. TUNEL-positive endothelial cells were counted as all cells in the overlaid image, above the internal elastic lamina which appear yellow. Percent apoptotic aortic endothelial cells were quantified in ratio as number of TUNEL-positive endothelial cells: number of endothelial cells.

4.0. DISCUSSION

The results of the present study suggest that hHcy has a causal relationship in the development of hypertension and furthermore, if untreated could lead to the onset of heart failure. At the cellular level, Hcy appears to exert its hypertensive pathological role by altering EC function. Indirectly Hcy has been implicated in the pathogenesis of hypertension by increasing oxidative stress. These studies focus primarily on Hcy impaired endothelium-dependent vasodilation (Chambers, Obeid, & Kooner, 1999). Homocysteine is thought to cause endothelial dysfunction and impair vasoreactivity by several means: (1) by generating ROS which quench NO bioavailability (Weiss, 2005), causing EC lipid peroxidation (Kaul, Zadeh, & Shah, 2006) and apoptosis (REF); (2) by causing eNOS uncoupling through the production of ADMA (Austin, Lentz, & Werstuck, 2004); (3) by increasing EC inflammatory mediators such as MCP-1 and IL-8 (Podder, et al. 2001) and (4) by altering the production and/or bioavailability of other vasoreactive mediators such as ET-1 and prostacyclin (Demuth, et al., 1999). There is also the view that the relationship between elevated Hcy and the development of hypertension is confounded by many other variables which dismiss Hcy causal association. Impaired renal function is shown to increase both BP and elevated Hcy levels (Stehouwer & van Guldener, 2003). Sutton-Tyrrell et al. (1997) demonstrated that the causal association between Hcy levels and hypertension was independent of renal failure, by excluding patients with impaired renal function from their correlations. Smoking and alcohol consumption are known causes of essential hypertension but are not related to Hcy levels (Lim & Cassano, 2002). Changes in renal function and dietary/lifestyle factors could also have an association with hHcy and the development of hypertension. Yet, when vitamins B₁₂ and B₆ are administered as an agent to lower Hcy levels, BP also decreases, independently of any further changes in renal function or lifestyle factors (van Dijk, et al., 2001).

4.1. HIGH-METHIONINE DIET INDUCED INCREASES IN FASTING PLASMA HOMOCYSTEINE LEVELS

Robin et al. (2004) demonstrated that a methionine-enriched diet induced mild hHcy and hypertension in rats. The standard rat diet that was fed to the control animals in this study contained a base concentration of 0.33% (w/w) L-methionine. After feeding the animals ad

litium an additional 1.7% (w/w) L-methionine for 2 weeks, Hcy fasting plasma concentrations were approximately 1.5 fold higher than age-matched controls ($5.52 \pm 0.29 \mu\text{mol/L}$ vs. $4.32 \pm 0.16 \mu\text{mol/L}$) and by 8 weeks of feeding, they remained significantly elevated as compared to age-matched controls ($7.22 \pm 0.24 \mu\text{mol/L}$ vs. $4.93 \pm 0.24 \mu\text{mol/L}$). These results are very similar to the Hcy concentrations measured by Robin et al. (2003) which found non-fasting Hcy levels were elevated 2-3 fold, compared to controls, when feeding WKY rats a 1.45% DL-methionine diet for 10 weeks. The rats in the present study were fasted for at least 8 hours, overnight to exclude any methionine loading effects on plasma Hcy concentrations. Oral methionine loading will produce an acute rise in plasma Hcy by 2 hours, with a peak Hcy response at 8 hours and return to baseline by 24 hours post loading (Chambers, Obeid, & Kooner, 1999). The fasting plasma Hcy concentrations in this report therefore represent overall basal levels that the rats develop throughout the study. These levels are lower than those normally associated with hHcy ($> 15 \mu\text{mol/L}$) but are significantly higher than controls. The significant increase in fasting plasma Hcy concentrations provides an evidence based approach for making experimental conclusions associated with Hcy activity.

The linear regression of methionine diet exposure to fasting plasma Hcy concentrations indicates that the prolonged feeding of a MD diet would progressively increase plasma Hcy concentrations. Zhang et al. (2004) found that SD rats fed an additional 1% DL-methionine in their diet for 14 weeks also had fasting plasma Hcy concentrations 2-3 fold higher than control. The trend is that a diet rich in methionine affects Hcy metabolism. In a study by Rowling et al. (2002), high L-methionine diet supplementation between 0.5 to 1% (w/w), up-regulated hepatic glycine N-methyltransferase, which regulates the ratio of SAM:SAH, causing continued methionine conversion into Hcy. Long-term feeding of MD may be responsible for up-regulating the conversion of Hcy back to its precursor methionine or to cysteine, explaining the mild response seen in this study (Zhang, et al., 2004). Use of a high-methionine diet is a reliable and affordable experimental means of increasing fasting plasma Hcy levels by 2 weeks of feeding.

4.2. **HEMODYNAMIC RESPONSE TO DIFFERENT INHALATION ANESTHETICS**

The initial studies were conducted using halothane as the inhalation anesthetic to maintain surgical anesthesia during hemodynamic recordings in rats. Halothane was discontinued, due to its associated hepatotoxicity and was not available for subsequent studies. The anesthetic was changed to isoflurane, which is resistant to biodegradation and tissue uptake (0.17% of isoflurane appears as urinary metabolites), explaining its minimal toxicity (Eger, 1984). Since hemodynamic measurements had been conducted on both halothane and isoflurane anesthetized animals, a comparison was necessary to evaluate if the results differed due to the anesthetic used. We found that, control animals under isoflurane vs. halothane anesthetic had significantly higher LVSP and LVDP. There was no change in isoflurane arterial pressures when controls or MD treated rats were compared and it is known that isoflurane does not increase myocardial contractility (Nathan & Odin, 2007). Also, Eger (1984) suggests that isoflurane decreases TPR and thus arterial pressures, confounding the results of this study, where increased afterload/LVSP was shown in the isoflurane group. Rivenes et al. (2001) showed that isoflurane produced no significant change in either ejection fractions or shortening fractions of the LV, both expected to be only mildly affected by increased afterload, as measured by echocardiograph. Whereas, halothane caused a significant decrease in both ejection and shortening fractions of the LV, attributed to its negative inotropic effects (Rivenes, et al., 2001).

Control animals anesthetized with halothane were shown, in this study, to have markedly higher MAP and DP than the isoflurane anesthetized controls. In contrast, Rivenes, et al. (2001) compared MAP under both halothane and isoflurane and found the most significant depression in the halothane group. Their results were further corroborated by a decrease in vascular resistance during both halothane and isoflurane treatment (Rivenes, et al., 2001). The increase seen in the halothane group MAP in this study is believed to be a direct reflection of the marked increase in DP. Increased arterial DP should correspondingly increase afterload, and therefore LVSP. Given the claim by Dale and Brown (1987), that myocardial depression and cardiac arrhythmias are the major CV disadvantages of using halothane and that it is known that halothane is a negative inotropic agent, it is presumable that halothane caused a marked increase in afterload, seen as an increase in arterial DP, and also reduced myocardial contractility, impairing the reactivity of the LV pressure-volume loops, resulting in decreased LVDP and LVSP.

Isoflurane also caused a significant elevation in the rate of myocardial contractility in control rats as compared to those anesthetized with halothane. It is known that halothane has a greater negative inotropic effect than isoflurane and furthermore has been demonstrated to preserve HR, opposed to isoflurane which has positive chronotropic effects (Rivenes, et al., 2001). Furthermore, isoflurane has also been demonstrated to increase both myocardial excitability and intraventricular conduction, both with the potential to increase HR (Nathan & Odin, 2007). An increase in the rate of contractility can also increase HR, suggesting the results of this study are in accord with those of Nathan and Odin (2007).

Lastly, the effect of MD on isoflurane and halothane hemodynamic measurements were very different. The LVSP increase seen in control animal under isoflurane anesthetic was reversed with MD treatment such that the animals on the MD under halothane had significantly higher LVSP than their matched isoflurane counterparts, but had no effect on LVDP or LVEDP. Furthermore, the MD diet had no effect on rate of myocardial contractility in either anesthetic group compared to controls. However, at 4 weeks MD exposure, the rate of myocardial relaxation was increased in both the isoflurane and halothane groups compared to their respective anesthetic controls. Other studies have shown that prolonged hHcy causes structural changes to the LV eventually resulting in impaired diastolic and systolic function corroborated by low LV ejection fractions (Cesari, et al., 2004). Reduced LV ejection causes an increased preload, which by the LV pressure-volume loops should result in more forceful ventricular contraction as to improve systolic function. Increased LVSP in response to Hcy dependent decreases in ejection fractions, were reflected as an increase in arterial SP seen in the halothane group after 4 weeks MD exposure. The relationship between short term hHcy and LV systolic function is not well described in the literature. At 4 weeks of MD exposure, changes to the myocardium and vasculature may be in their infancy, requiring this study to investigate extended periods of elevated plasma Hcy levels.

The effects of halothane and isoflurane anesthesia on hemodynamic parameters of male WKY rats are not comparative. Halothane produces a marked decrease in the myocardial index of contractility demonstrated by a significantly increased LVSP and LVDP, known to be caused by its associated negative inotropic effects. Isoflurane is a current inhalation anesthetic of choice as it does not produce the same level of LV depression as compared to the discontinued

Halothane. Therefore, the results of this study are not comparable and isoflurane is recommended for further studies.

4.3. HEMODYNAMICS OF RATS WITH ELEVATED PLASMA HOMOCYSTEINE

By 8 weeks of feeding MD to WKY rats, all measures of arterial pressure were significantly elevated, as were LVSP and LVEDP, all marked by a sudden decrease in the rate of myocardial contractility. Very similar to the findings of Robin et al. (2003), MD increased the systolic pressure of WKY rats. Robin et al. (2003) found that systolic pressures in WKY rats, as measured by tail-cuff, were significantly elevated by 5 weeks of MD and were sustained until the end of the study at 10 weeks. The baseline systolic BP attained by their group was between 100 – 110 mmHg, similar to this study measured under isoflurane anesthesia where systolic BP was 98.9 – 107 mmHg. Hcy dependent increases in SP were higher measured by tail-cuff by Robin et al. (2003) (>140 mmHg) than this study (126 mmHg). Other studies have also found a correlation between MD exposure and elevations in arterial BP. Rolland et al. (1995) observed both systolic and diastolic hypertension in minipigs fed a methionine-rich diet for 4 months. In human patients, elevations in total Hcy plasma concentrations either have a strong, independent association with systolic hypertension (Sutton-Tyrrell, et al., 1997) or they are associated with elevated MAP and DP (Fiorina, et al., 1998). Supporting all findings is the documented evidence that hHcy increases TPR and BP by causing endothelial injury and dysfunction and modifying vascular wall compliance through structural changes (Huang, et al., 2008).

Interestingly, due to the complexity of CV regulation and the development of essential hypertension, the effects of MD on LV pressures may be caused by or causal to the overall increases seen in arterial pressure. This study showed that LVSP increased incrementally with prolonged exposure to MD. Since the literature available regarding LV pressure responses to hHcy are very limited, physiological explanations corroborated by the hemodynamic measurements in this study will be discussed using LV pressure-volume loops. It is well documented that prolonged hHcy leads to left ventricular systolic and diastolic failure (Cesari, et al., 2004) (Bokhar, et al., 2005) (Devi, et al., 2006), but the progressional development of such failure has yet been elucidated.

The progressive increase in LVSP, seen in the MD treated rats for 8 weeks is correlated with increased MAP, SP and DP. As per LV pressure-volume loops, increased arterial pressure

is equal to an increase in afterload. Increased afterload, if inotropy is constant, may cause an increase in LVSP and end-systolic volume, being consequential to a decrease in SV, and a small increase in end-diastolic volume. At 8 weeks of MD exposure LVEDP was significantly elevated in association with the marked increase in LVSP. Van der Velde, et al. (1991), using aortic impedance as a model of increased afterload in canines, proved that examining end-systolic pressure-volume relationships which include an increased rate of myocardial contraction and stroke work compared to end-diastolic volumes are sensitive enough to be interpreted as increased LV contractility. Therefore the results of the present study may indicate the development of heart failure because even as LVSP increases in response to higher afterload, LVEDP also increases higher than would expected, corresponding to increased preload and therefore compromised LV ejection. However, further changes in $\pm dp/dt$ would corroborate these findings. Preload is defined as the greatest pressure reached in the ventricle during diastolic filling and is determined by venous return, atrial systolic pressures, and ejection fractions, the amount of blood ejected from the previous ventricular systole (Greenway & Loutch, 1986). Given the results of van der Velde et al. (1991), if these markers of LV function were also correlated to increase inotropy, and LVEDP is elevated, then the LV ejection fraction is likely significantly reduced. Furthermore, LVDP was unchanged at 8 weeks MD exposure as compared to control animals in this study. The LVDP is the maximal pressure achieved in the LV during systole, equal to the LVSP subtracted by the LV diastolic pressure. The increase in LVEDP, explained by a decrease in ejection fraction and incomplete LV emptying would correspondingly increase LV diastolic pressure. Also given the increase seen in LVSP, the overall LVDP is offset by the increase of both parameters simultaneously. Cesari et al. (2005) showed that hHcy was inversely related to LV ejection fractions and associated most strongly in arterial hypertensive patients. The results of this study show that arterial hypertension in hHcy may either unmask or favor the onset of low LV ejection and predispose the animals to subsequent heart failure.

Another indication of the development of hHcy induced heart failure are the changes seen in the myocardial indices of contractility and relaxation. At 4 and 6 weeks MD exposure – dp/dt and + dp/dt increased significantly from the young control group. However, at 8 weeks MD, – dp/dt and + dp/dt decreased. The trend appear to indicate an increase in myocardial contractility and relaxation up to the point of increased arterial pressures and premature cardiac failure, which

may coincide with 8 weeks of MD exposure in this study. Devi et al. (2006) showed $+ dp/dt$ and $- dp/dt$ were significantly lower in rats after 10 weeks of MD feeding. Rosenberger et al. (2010) demonstrated that mice fed a Hcy-enriched diet for 12 weeks had significantly reduced LV shortening, indicating a decrease in contractility and relaxation of cardiomyocytes. Moreover, Rosenberger et al. (2010) also showed that the mice referred to previously had significantly prolonged QRS complexes which are related to atrioventricular conduction delay and can result in marked end-diastolic volume or preload increases. The QRS complex is related to the time it takes for action potential conduction through the ventricle, and begins when the heart is in atrial systole and isovolumetric contraction, just prior to the opening of the aortic valve and ventricular systole. In this study, these results were replicated with respect to increased LVEDP which also occupies the same time frame as the QRS complex. This effect can be compensated for by increasing HR, which should restore CO despite ventricular failure. Furthermore, the significant decrease in $+ dp/dt$ at 8 weeks MD is also confirmed by Rosenberger et al. (2010) such that when corrected for HR, the QT interval of hHCy mice was also significantly prolonged, indicating decreased myocardial conductivity. The QT interval begins during atrial systole and ends at the end of ventricular contraction. Therefore, any disturbance seen in $+ dp/dt$ may be attributed to changes during this period of time. A prolonged QT interval is very dangerous as it can quickly result in ventricular fibrillation which causes sudden death if not treated (Morita, Wu, & Zipes, 2008). However, the decrease in myocardial relaxation may be due to altered calcium management or to a direct myocyte effect caused by Hcy (Perreault, et al., 1990). The results of this study are in accord with others with respect to impaired myocardial conductivity and generation of myocyte contractility again predicting resultant cardiac failure after prolonged hHcy, in association with elevated arterial and LV pressures.

The most interesting aspect of the results of this study are the changes seen in $+ dp/dt$ and LVSP prior to detectable increases in arterial BP. At 4 and 6 weeks of MD exposure, the LV appears to be reacting to increased preload. The developed pressure in the ventricle is higher at 6 weeks of MD and the rate of myocardial contractility increases up to 6 weeks as well. These results indicate that as per the Frank-Starling Law of the Heart and its associated LV pressure-volume loops, as LV SV increases, so does CO and arterial pressure. So at 8 weeks MD, arterial pressures are significantly elevated, potentially caused by cardiac responses not by changes to the vasculature.

At 8 weeks of MD feeding, detrimental changes to LV hemodynamics were becoming apparent. It is expected that the myocardial indices of contractility and relaxation should have been more responsive to the changes seen in LVEDP and LVSP, however, it is believed that if the study was continued into longer exposure periods, the development of heart failure would be even more evident. Furthermore, arterial blood pressure was significantly elevated, which as defined indicates the animal were hypertensive. The following studies elucidate whether the development of hypertension was due to changes in the vasculature, increased TPR, or if the developing changes to LV hemodynamics caused reflective BP increases, and by what mechanism oral heparin was able to attenuate some of the effects.

4.4. EFFECT OF ORAL HEPARIN TREATMENT ON THE HEMODYNAMICS OF RATS WITH ELEVATED PLASMA HOMOCYSTEINE

Heparin was orally administered to WKY rats in conjunction with MD feeding for 8 weeks. Hemodynamic measurements were collected under isoflurane anesthesia at the end of the 8 week cycle in heparin plus MD exposed rats, MD only exposed rats and controls. Firstly, heparin treatment did not effect fasting plasma Hcy concentrations. Total fasting plasma Hcy concentrations were elevated in both the MD and MD plus heparin treated rats as compared to controls. Blood samples for the determination of fasting total Hcy concentrations were collected in cooled vials containing EDTA, a common laboratory anticoagulant which irreversibly binds calcium ions, a crucial element of the coagulation cascade, then centrifuged to permit testing of plasma fractions. Nybo et al., (1972) demonstrated that after accounting for plasma dilution due to erythrocyte water loss, NaF/Heparin use as an anticoagulant for blood collection with the purpose of determining total plasma Hcy, produced identical results as those sample collected with EDTA. The results of this study and Nybo et al., (1972) both indicate that heparin has no metabolic or catabolic effect on Hcy. Therefore, as heparin treatment does not affect Hcy levels, its use in the present study can be associated as that of an agent which affects *in vivo* hemodynamic and biochemical results independent of elevated plasma Hcy levels.

The results of this heparin study utilized different WKY rats than those from the effects of Hcy alone study previously discussed. Validating the results of the previous section, the hemodynamic measurements of 8 week MD and control WKY rats were reproduced almost identically in this heparin study. The most notable hemodynamic events seen in this study were

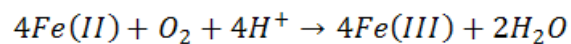
that all measures of arterial pressure were significantly elevated by 8 weeks of MD feeding, as were LVSP and LVEDP compared to control. Interestingly, heparin appears to have attenuated the increases seen in all of the aforementioned pressures. It was previously discussed that the hemodynamics of the 8 week MD fed rats indicated changes to (1) a presumed increase in TPR, with respect to increased ventricular afterload/LVSP; (2) increased arterial pressures, with respect to the increase in ventricular preload/LVEDP; (3) reduced ejection fraction, again with respect to increased preload/LVEDP; (4) and the onset of heart failure with respect to decreased $+ dp/dt$. It is plausible that the increase in afterload/LVSP and subsequent increase in LVEDP and arterial pressures of rats fed MD in this study is attributed to an increase in TPR as caused by endothelial dysfunction, inhibited by heparin.

The hypertensive effects of hHcy are strongly correlated to increased TPR and endothelial dysfunction (Chambers, Obeid, & Kooner, 1999). Chambers et al., (1999) have shown that increments of 2 – 3 $\mu\text{mol/L}$ plasma Hcy, similar to the increases seen in this study, attained by low-dose oral methionine feeding was enough to induce endothelial dysfunction. The increase in arterial pressures at 8 weeks of MD feeding are likely correlated to the increases seen in both preload/LVEDP and afterload/LVSP. The ability of heparin to prevent the previous hemodynamic increases may be intrinsically linked to its ability to protect against endothelial cell death and dysfunction (Hiebert & Liu, 1990) and consequently increases in TPR and BP. Hyperhomocysteinemia dependent endothelial dysfunction is characterized by reduced eNOS activity (Lee, et al., 2004). Reduced eNOS expression is associated with impaired endothelium dependent vasodilation, and thus is correlated with increased TPR. Heparan sulfate proteoglycans (HSPGs) due to their high negative charge, serve as an important anchor for many external molecules on the cellular surface, making them an integral partner in the recruitment and activation of signaling pathways (Bishop, Schuksz, & Esko, 2007). Ramella et al. (2010) demonstrated that vasostatin 1, a vasodilating and cardiac stabilizer during sympathetic stress responses, requires HSPGs to anchor and endocytose into endothelial cells where it initiates eNOS activation. Their observations were further confirmed, as treatment with heparinase completely prevented vasostatin 1 endocytosis into endothelial cells (Ramella, et al., 2010). If heparin increased NO production, it is reasonable that levels of S-nitrosohomocysteine would also increase. Adducts between NO and Hcy have been shown to actually stabilize NO, enhancing its vasodilatory effects 16 fold over S-nitrosocysteine (Gow, Cobb, & Stamler, 2001).

Another potential mediator of Hcy dependent increases in LVSP, LVEDP and arterial pressures is endothelin-1 (ET-1), a very potent vasoconstrictor and potential mediator of cardiac hypertrophy (Kedzierski & Yanagisawa, 2001). Endothelial dysfunction has been observed with respect to increased levels of ET-1 in hHcy demonstrated by significantly impaired endothelium-dependent vasodilation, independent of oxidative stress (Tousoulis, et al., 2010). Tousoulis et al. (2008) further indicated that the Hcy dependent rise in ET-1 levels was associated with fasting plasma total Hcy levels, and not with acute-methionine loading and transient plasma Hcy increases. Heparin has several demonstrated effects on ET-1. In cultured bovine endothelial cells, heparin suppressed ET-1 release (Imai, Hirata, & Marumoand, 1993). Kuwahara-Watanabe et al. (2005) further described that heparin suppresses the gene expression of ET-1 at the level of transcription. Heparin and HSPGs are again noted to act as important anchors on the endothelial surface facilitating the binding of another key factor known to regulate BP through ET-1. Heparin-binding epidermal growth factor (HB-EGF) has been described as having a plethora of protective effects, especially concerning organ microcirculation. Zhou, Brigstock and Besner (2009) demonstrated that HB-EGF increases pressure-induced vasodilation and reduces ET-1 vasoconstriction in adult rat mesenteric arterioles. Endothelin has very different effects depending on which receptor it is bound. The ET_A receptors are found in VSM and illicit vasoconstriction when activated. The ET_B receptors are located on endothelial cells, and cause the release of NO and subsequent vasodilation when bound by ET-1. Interestingly, heparin not only affects ET-1 release and synthesis, it also has effects on its receptor expression. For instance, in a rat model of cyclosporin nephropathy, heparin decreased ET-1 BP response in the left carotid artery by over 20% (Bennett, et al., 1997). The study by Zhou et al. 2009, demonstrated that HS-EGF upregulates ET_B receptor expression.

The most widely accepted hypothesis explaining the relationship between hHcy and the development of hypertension is that Hcy increases oxidative stress which is directly responsible for causing endothelial dysfunction. However, this hypothesis continues to be debated. For instance, Chao et al., (2000) showed that increases in Hcy concentrations, induced by methionine treatment, reduced endothelium-dependent vasodilation in the absence of increased oxidative stress. However, other studies have shown that elevated Hcy levels reduce endothelium-dependent vasodilation in association with increased superoxide production as well as increased protein nitrosylation, a biomarker for peroxynitrite formation (Li, et al., 2002). The evidence is

varied, but the oxidative stress hypothesis for hHcy could explain increases in arterial BP and increased TPR, affecting LVSP and LVEDP. Some of the results of increased oxidative stress in hHcy includes the reduced bioavailability of NO in a superoxide dependent manner producing peroxynitrite (Heydrick, et al., 2004), lipid peroxidation of endothelial cells (Loscalzo, 1996) and redox sensitive apoptosis of endothelial cells (Mercie, et al., 2000) both causing cell death and an associated reduction in NO availability. Ross et al., (1992) believe that heparin and heparan sulfates have direct antioxidant functions. Firstly, heparin is known to release EC-SOD from endothelial cell surfaces in humans and mice (Karlsson & Marklund, 1987), but these effects on EC-SOD in rats is limited since EC-SOD has a significantly lower heparin affinity (Faraci & Didion, 2004). The difference exists due to the lack of an amino acid, crucial for protein subunit interactions (Marklund, 1984). Because the rat essentially lacks heparin associated EC-SOD, this effect of heparin is excluded as a mechanism of antioxidant protection. However, heparin and other GAGs because of their charge, may structurally make them reservoirs for cations preventing their participation in ROS reactions (Grant, Long, & Williamson, 1987). Iron ($\text{Fe}^{3(2)+}$) catalyzes the Haber-Weiss reaction, which is a significant source of the hydroxyl radical, a known promoter of lipid peroxidation (Minotti & Aust, 1989). Halvorsen et al. (1996) have shown that low concentrations of Hcy ($< 6\mu\text{mol/L}$) promotes cation catalyzed LDL oxidation, whereas high concentrations of Hcy were protective *in vitro*. Other studies have shown a pro-oxidant effect of thiols in systems containing free Fe^{2+} (Lynch & Frei, 1997), and also an antioxidant effect in systems containing free Fe^{3+} (Lynch, Campione and Moore, 2000). In a histochemical study by Garcia-Segura (1977), it was shown that Fe^{2+} and Fe^{3+} readily bound to heparin-related GAGs, replacing Ca^{2+} from heparan sulphate. It is not clear whether iron bound to heparin is unable to participate in Haber-Weiss type reactions. However, the relatively low pH that surrounds a polyanion such as heparin, enables acidic oxidation without ROS production as per the following equation (Ross, Long, & Williamson, 1992):



(4.1)

There is accumulating evidence which indicates that antioxidants prevent endothelial dysfunction in hHcy. Vitamin C, an ROS scavenger was shown to prevent NO inactivation by superoxide (Kanani, et al., 1999), and potentiate ACh-induced vasodilation (Virdis, et al., 2001)

in hHcy. The antioxidant melatonin has also been shown to reduce Hcy dependent contraction on the human umbilical artery, thus decreasing TPR (Okatani, Wakatsuki, & Reiter, 2000). All of this evidence indicates that if heparin were to act as an antioxidant in the assumed pro-oxidant vascular atmosphere of hHcy, then it may be responsible for inhibiting endothelial dysfunction, shown by decreased LVSP, LVEDP and arterial pressures.

Systemic characterization of heart failure includes mechanisms of cardiomyocyte stress such as apoptosis, ischemic injury, and inflammatory reactions (Kitahara, et al., 2010). Moreover, hHcy has been characterized as causing LV hypertrophy, myocardial fibrosis and coronary remodelling, subsequent to the cardiomyocyte stressors listed previously (Joseph, Washington, & Joseph, 2003). Heparin has been described as a protective agent against all three proposed cardiomyocyte stressors and therefore against end-stage LV dysfunction remodelling as associated with hHcy. Since it is not exactly known which happens first, vascular or cardiac dysfunction, all of the aforementioned theories are useful when evaluating the CV effects of heparin on hHcy.

A high-methionine diet significantly increased LVSP and LVEDP by 8 weeks in this study and heparin treatment mildly attenuated the increase. The LV of the heart will hypertrophy as an adaptive response, when overloaded with increasing pressure and volume to preserve its function (Yajima, et al., 2005). Left ventricular hypertrophy is a strong predictor of CV morbidity and mortality in arterial hypertensive patients (Levy, et al., 1990). Plasma Hcy levels are independently associated with LV mass, wall thickness in the Framingham Heart Study (2004). The remodelling changes seen in hHcy hearts are correlated with increased collagen deposition and myocardial fibrosis, as seen in rats fed MD for 10 weeks (Devi, et al., 2006). If the structural changes in the LV were progressive up to 8 weeks of MD feeding, then heparin's ability to prevent the increases seen in LVSP and LVEDP may be related to protecting the LV from hHcy dependent remodelling. Yajima et al. (2005) found that when heparin and fibroblast growth factor-2 (FGF-2), a heparin-binding protein that induces angiogenesis, are injected into the myocardium of rats with heart failure, that myocardial shortening and therefore LV ejection fraction, as well as the heart-to-body weight ratio were significantly improved. Brain natriuretic peptide (BNP) is a sensitive marker for heart failure, secreted in response to increased ventricular wall tension reflecting changes in ventricular function another marker of heart failure (Yasuda, et al., 1999). Atrial natriuretic peptide (ANP) is released only during LV

hypertrophy in rats (Yajima, et al., 2005). Yasuda et al. (1999) and Yajima et al. (2005) demonstrated that heparin treatment decreases BNP alone or both ANP and BNP respectively. Furthermore, Yajima et al. (2005) correlate heparin and FGF-2 activity, known to release NO and be anti-apoptotic, as being responsible for normalizing systolic LV wall stress and decreasing myocardial stiffness, allowing for greater muscle contraction at a given preload (Matsubara, et al., 1998). These results indicate that heparin may have prevented an increase in LVEDP and LVSP by inhibiting or retarding the progressive remodelling of the LV cause by elevated plasma Hcy levels.

Another finding of this study was a significant increase in LVDP in MD fed rats treated with heparin as compared to controls, but the increase in LVDP in the diet group alone failed to be significant. The developed pressure of the LV is an indication of the relative increases to both LVSP and LV diastolic pressure. Because LVSP and LVEDP were both significantly elevated in the MD group, this offset any increase in LVDP explained by reduced ejection fraction, incomplete LV emptying and therefore increased diastolic pressure. In the heparin group however, the small increase in LVSP (119.9 mmHg) as compared to control (112.3 mmHg) was likely exacerbated by a presumable decrease in LV diastolic pressure, resulting in a significantly elevated LVDP (111.4 mmHg) compared to controls. The diastolic pressure in the heparin group could be related to an overall improvement in systolic and diastolic function if indeed LV hypertrophy and fibrosis were inhibited.

Lastly, there were no significant changes seen in the myocardial indices of contraction or relaxation in this heparin study. In the earlier study, MD feeding by 8 weeks significantly decreased $+dp/dt$ compared to controls. The trend is similar in this study however insignificant. Additionally, heparin appears to increase $+dp/dt$ back to control levels, again insignificantly. As previously discussed, the negative changes seen in $+dp/dt$ are likely correlated to the onset of heart failure in the MD fed rats.

Because oral heparin treatment was able to attenuate the development of hypertension in the MD fed rats, and it is known to be endothelium protective, it is believed that the homocysteine induces changes in hemodynamic parameters is caused by endothelial dysfunction and subsequently increased TPR. Changes seen to the hemodynamic parameters of the LV were consequential of the initiating increases in arterial BP. Prolonged investigation of the diet model used in these experiments would reveal the development of heart failure, seen as further

compromised LV ejection fractions and increased LVEDP and changes in the myocardial indices of contractility and relaxation.

4.5. ELEVATED PLASMA HOMOCYSTEINE AND LIPID PEROXIDATION

Malondialdehyde (MDA) is a product of lipid metabolism and is used as an experimental biomarker of oxidative stress. Domagala et al., (1997), Hcy levels are positively correlated with MDA levels. However, in their study, the lowest concentration of Hcy tested in conjunction with MDA levels was $> 30 \mu\text{mol/L}$ and MDA levels were maximal at 1 mmol/L , which is beyond realistic physiological concentrations (Domagala, Libura, & Szczeklik, 1997). In a similar study, Ventura et al. (2000) found that only acute increases in plasma Hcy after methionine loading caused significant increased MDA levels. The peak oxidative damage seen by Ventura et al. (2000) occurred eight hours post-methionine load. Heydrick et al. (2004) measuring isoprostane levels as a marker of lipid peroxidation, showed that Hcy dose dependently increased total isoprostanes with a discernable effect at $50 \mu\text{mol/L}$ Hcy, and significant observations at $300 \mu\text{mol/L}$ Hcy. Weiss et al. (2003) indicate that in a stereospecific manner, only the L-isof orm but not the D-isof orm of Hcy increases total isoprostane levels in endothelial cell cultures. Weiss et al. (2003) have also shown that loading endothelial cell cultures with catalase had no effect on lipid peroxidation, excluding the role of hydrogen peroxide in the process. Dudman et al. (1991) and Wall et al. (1980) both show that hHcy induces hydrogen peroxide dependent oxidative damage to endothelial cell cultures.

Many of the previous findings were made at non-physiological levels of plasma Hcy, rendering them unrealistic for comparison with this study. The results of the present study were performed in fasted animals, with significantly lower total plasma Hcy concentrations than peak levels, seen eight hours after methionine loading (Chambers, Obeid, & Kooner, 1999). Also at this time it is entirely probable that MDA was already removed from the serum or tissue samples either by oxidation, or by forming protein adducts (Farmer & Davoine, 2007). Rat tissue MDA levels reported in the literature range from $0.3 - 10 \text{ nmol/mg}$ (Ataie, et al., 2010) (Kiziler, et al., 2008), which are in agreement with this study. High-methionine diet feeding did not appear to significantly increase MDA concentrations in serum, LV, kidney, mesentery or aortic samples in this study. However, there is a trend towards decreased tissue MDA levels in the MD and MD plus heparin group as compared to control tissues. There are several explanations for this trend.

Firstly, the trend may be due to physiological and heparin dependent detoxification and elimination of free Hcy from the blood. Total plasma Hcy concentrations comprised of approximately 80% protein bound Hcy and 2% free Hcy (Jakubowski, 2004). The free fraction of Hcy is the most biologically active form. Given the already very low concentrations of Hcy in this study after fasting, the residual free fraction of Hcy would be very minimal. Furthermore, the free fraction of Hcy is readily available for sequestration into further protein adducts, Hcy-thiolactone, or even S-nitrosohomocysteine. S-nitrosohomocysteine is an adduct between NO and Hcy, which may be a detoxifying mechanism for protection against endothelial cell lipid peroxidation. Fu et al. (2002) claim that endothelial cells will increase NO production facilitating the formation of the adduct, thereby reducing the bioavailability of Hcy. Heparin would further potentiate this effect as it is shown to anchor vasostatin-1, encouraging eNOS activation (Ramella, et al. 2010). S-nitrosohomocysteine has been shown to be protective in many ways against Hcy toxicity including the prevention of endothelial cell apoptosis and the proliferation of VSM cells (Stamle, et al., 1993). Similarly, Lee et al. (2005) have shown that the NO donor, s-nitroso-N-acetylpenicillamine (SNAP) increases NO levels *in vitro* forming S-nitrosohomocysteine, thereby inhibiting ROS generation of Hcy sulfhydryl groups and furthermore inhibited Hcy dependent MDA increases. Therefore, it is reasonable that due to the reduced bioavailability of free Hcy, either by fasting or adduct formation, that MDA levels should be similar to controls, which were seen in this study.

High-methionine diet feeding significantly increased MDA levels in liver samples compared to heparin treatment and significantly decreased levels in kidney samples compared to controls. Hepatic uptake of Hcy happens readily and therefore may act as a sink for Hcy. The increase seen in liver MDA is likely related to its overall exposure to Hcy. Heparin, acting to encourage Hcy adduct formation reducing its bioavailability, may have attenuated the increase in MDA levels. The significant decrease of MDA levels seen in the kidneys of the MD group as compared to controls, and insignificant decrease compared to the heparin treated group, may be due in part to the generation of thiol antioxidants, inhibiting lipid peroxidation. The kidneys are capable of Hcy transsulfuration, producing cysteine and GSH. N-acetylcysteine, a derivative of L-cysteine, has been suggested to be a very powerful antioxidant, alone or by acting as a precursor for GSH (Han, et al., 1997). Zembron-Lacny et al. (2009) have shown that N-acetylcysteine markedly decreases lipid peroxidation measured in healthy, male subjects. If

kidney exposure to elevated levels of Hcy increased its transsulfuration into cysteine, then it is likely that N-acetylcysteine levels may increase protecting the organ from lipid peroxidation. However, the aforementioned rationales are purely speculative. Therefore this study finds that there was no clear association between MDA and Hcy levels, and therefore the potential antioxidant effect of heparin could not be corroborated.

It should also be noted that the serum MDA results seen in this study are unusually high (20 – 30 nmol/ml) compared with the results of other studies using much higher Hcy concentrations (0.5 – 2 nmol/ml) (Domagala, Libura, & Szczeklik, 1997) (Ventura, et al., 2000). The marked increase in serum MDA levels as compared to similar studies is likely from sample contamination due to partial red blood cell (RBC) hemolysis. The serum samples collected for the MDA assay were drawn through a small gauge cannula inserted into the femoral artery after the hemodynamic study. The serum samples withdrawn from the collection tubes after centrifugation had a notable red colour, likely caused by RBC hemolysis during sampling. RBC are sensitive to membrane lipid peroxidation and the oxidation of hemoglobin (Melchiorri, Reiter, & Sewerynek, 1995). When RBCs are present in serum samples at very low levels, 1×10^8 RBC/ml, MDA concentrations increase significantly (Hooser, et al., 2000). Therefore, the results of this study are not suitable for comparison with others. However, because the literature thus far indicates that significant MDA levels in serum are attained only with moderate to severe hHcy levels, the probability of seeing any positive association was very unlikely. In conclusion, there was no direct evidence indicating that increased fasting plasma homocysteine levels caused increases in lipid peroxidation by measuring MDA levels.

4.6. ELEVATED PLASMA HOMOCYSTEINE AND GLUTATHIONE

Hempel (1998) suggested that hHcy sensitizes endothelial cells to oxidative stress by reducing glutathione levels. Chern et al. (2001) have also shown that folate-deficient human hepatoma cells had suppressed intracellular GSH content. Prolonged exposure to MD significantly decreased plasma GSH levels as compared to control. There also appears to be a trend towards heparin restoring plasma GSH levels. The concentration of reduced glutathione (GSH) in control plasma samples measured in this study were 4.24 ± 0.94 $\mu\text{mol/L}$, which are in accord with other findings that report a range from 2.14 – 6.37 $\mu\text{mol/L}$ GSH in healthy human plasma (Mansoor, Svardal, & Ueland, 1992). The redox ratio of GSH to oxidized glutathione

(GSSG) is an important indicator of oxidative stress. In its reduced state, GSH readily acts as a proton donor to detoxify hydrogen peroxide. The redox activity of GSH is dependent on glutathione peroxidase (GPx) which catalyzes the conversion of reduced GSH to GSSG. A decrease in GSH levels can therefore be indicative of increased ROS scavenging. However, this rationale would be more conclusive if GSSG levels were available for comparison. More commonly, studies indicate that hHcy reduces the function and expression of GPx, hindering the glutathione cycle thereby increasing oxidative stress (Lubos, Loscalzo and Handy, 2007) (Robin et al., 2004) (Jakubowski, 2004). Inhibition of GPx is associated with free Hcy concentrations of 1 – 5 $\mu\text{mol/L}$ (Chen, et al., 2000). The free Hcy concentrations in this study were maximally estimated at 0.2 $\mu\text{mol/L}$, based on the estimation of free Hcy being approximately 2% of the total fraction, making GPx inhibition unlikely. Regardless of the mechanism, the significant decrease seen in plasma GSH levels in MD fed rats is convincing of increased Hcy induced oxidative stress.

Furthermore, it is very interesting that heparin appears to be attenuating the decrease in GSH levels. In the LV, we found that GSH concentrations were significantly elevated in the heparin treated group as compared with the MD fed rats. Additionally, in the kidney, there was a non-significant trend towards an increase in GSH concentrations in the heparin treated group as compared to the MD and control rats. Singh et al., (2008) reported LV GSH concentrations of hHcy rats equal to 0.30 $\mu\text{mol/mg}$ protein, which are similar to the findings in this study. They have also shown that mast cell stabilizers prevent cellular heparin release and changes in intracellular LV GSH concentrations (Singh, Singh, and Balakumar, 2008). There is a lot of new interest in the role of the multidrug resistance protein (MRP1) in the regulation and transport of GSH. Multidrug resistance proteins function as ATP dependent anion export pumps (Ballatori, Krance, Marchan, & Hammond, 2009). The substrate specificity of MRP1 for GSH compared to GSSG is approximately 1:100, indicating that GSSG is preferentially exported by MRP1 after GSH oxidation (Leier, et al., 1994). Angelini et al. (2007) have shown that unfractionated heparin treatment in human leukocytes enhances glutathione export via MRP, in a non-oxidative environment. This could explain the increased serum GSH concentrations in the heparin treated group. The results of this study indicate that elevated Hcy levels induced oxidative stress, and since it is not believed that GPx activity was reduced, then GSSG levels would increase, as would their export in the presence of heparin. In this scenario, GSH levels

would decrease initially because of ROS detoxification, however, synthesis of new GSH would ensue, given the higher levels of Hcy. As per Chang (2007), MRP1 expression is high in the kidneys, cardiac muscle and erythrocytes, again potentially corresponding to the elevated GSH concentrations seen with heparin treatment in those areas.

Oral heparin treatment attenuated the decreases seen in GSH concentrations most tissues caused by elevated fasting plasma Hcy levels. This suggests that heparin can either ameliorate tissue antioxidant status by detoxifying ROS directly or by encouraging antioxidant formation. Both mechanisms would be endothelium protective in a pro-oxidant atmosphere, suggested to be the pathological mechanism of hHcy.

4.7. ELEVATED PLASMA HOMOCYSTEINE AND ENDOTHELIAL CELL APOPTOSIS

It is widely accepted that hHcy causes endothelial cell dysfunction, and recently it is believed to be partially associated with Hcy induced endothelial cell apoptosis. Endothelial function is dependent on a balance between pro- and anti-apoptotic signals, and disruptions to this system causes the pathogenesis of vascular disease (Stefanec, 2000). Lee et al. (2005) have shown that Hcy-mediated cell death of human umbilical vein endothelial cells displayed typical apoptotic features which included nuclear fragmentation and chromatin condensation, and that co-treatment of apoptosis inhibitors decreased the appearance of the classic morphological changes, confirming that Hcy caused endothelial cell apoptosis. In the this study, elevated total Hcy concentrations significantly increased the percent of apoptotic endothelial cells in the aorta and superior mesenteric artery of MD fed rats. Additionally, heparin treatment significantly prevented the apoptosis of mesenteric endothelial cells and appeared to decrease the number of apoptotic aortic endothelial cells in the current study. Firstly, apoptosis can be triggered by physical (radiation, mechanical damage), chemical (ROS, pharmaceuticals) or biological (receptor-mediated) signals (Freyssinet, et al., 1999). It was predicted, as per the oxidative hypothesis for hHcy, that increased oxidative stress would induce endothelial cell apoptosis in MD fed rats. The TUNEL stain used to detect positive apoptotic cells, does not discriminate between effectors involved in the apoptosis process. However, many other studies have chosen to measure apoptosis by way of its associated effectors such as the caspases, cytochrome C of the Bcl-2/Bax family of apoptotic proteins (Freyssinet, et al., 1999). In a method similar to that of

Chern et al. (2001), this study shows that in conjunction with increased cellular apoptosis are decreased levels of GSH, implicating oxidative stress as the primary mediator of Hcy dependent apoptosis. In the model by Chern et al. (2001), increased Hcy concentrations induced apoptosis in a process dependent on hydrogen peroxide formation. Their study indicated that hydrogen peroxide activated nuclear transcription factor kappa B, which facilitates the expression of pro-apoptotic genes (Chern, et al., 2001). Lee et al. (2005) also demonstrated that Hcy, in an ROS dependent manner, causes caspase-induced apoptosis in endothelial cells.

It was seen that the most significant decreases in GSH concentration, due to elevated Hcy levels were in the LV and plasma. Correspondingly, the amount of apoptotic endothelial cells in the aortic tissue of MD fed rats was significantly elevated. These results indicate that the redox capacity of the plasma and LV were compromised by decreased GSH, and the aorta, already under high-mechanical stress, would be the first site to encounter increased ROS, thereby having a greater risk for ROS dependent endothelial cell apoptosis. The superior mesenteric artery branches off of the abdominal aorta, prior to the renal artery. Similarly to the aorta, the superior mesenteric artery would be the second tissue, investigated in this study, to be exposed to increased ROS coming from the LV and plasma, thereby explaining its significant number of apoptotic endothelial cells. Lastly, there was no difference in the percent of apoptotic endothelial cells between all treatment and control groups in the renal artery, also branching off of the abdominal aorta, inferior to the mesenteric artery. Correspondingly, the GSH levels measured in the kidneys were unchanged by MD feeding, and thus the redox potential of this tissue, was not compromised and therefore likely not subjected to oxidative stress induced endothelial cell apoptosis.

There is a wealth of evidence indicating that NO mediates apoptosis. Firstly, low levels of endogenous NO production have been shown to prevent oxidative stress induced endothelial cell apoptosis through s-nitrosylation (Kwon, et al., 2001). Furthermore, Lee et al. (2005) confirmed that endogenous NO production interacts with Hcy to form S-nitrosohomocysteine, suppressing ROS formation and apoptosis treated with 1 mmol/L Hcy. Lee et al. (2005) attributed 50% of NO-dependent inhibition of apoptosis to only a 25% ratio between S-nitrosohomocysteine and Hcy. This result indicates that the concentration of NO in the endothelium can be directly correlated with the pro-apoptotic, pathogenic activity of Hcy. Heparin has already been discussed in detail pertaining to its ability to increase NO production

through cell-surface facilitated transport of vasoactive substances. Therefore, heparin treatment may have increased NO production in the endothelium of the aorta and superior mesenteric bed protecting against apoptosis by either sequestering Hcy through the formation of S-nitrosohomocysteine, or by direct action on pro-apoptotic effectors such as the caspases or NF- κ B.

Elevated plasma Hcy levels caused endothelial dysfunction by increasing the amount of apoptotic endothelial cell death in vascular tissue samples. This evidence further corroborates the conclusion that changes in the hemodynamics of rats with increased Hcy levels were seen because of increased oxidative stress which was deleterious to the endothelium, causing increased TPR and arterial BP, overtime possibly leading to heart failure, seen in this study as increasing LVEDP and subsequent LVSP.

5.0. CONCLUSION

The results of the present study suggest that (i) a high methionine diet fed to male WKY rats significantly elevates plasma Hcy levels by 2 weeks of feeding. (ii) The hemodynamics of animals anesthetized with halothane versus isoflurane are significantly different, and therefore the results from each are considered inappropriate for comparison. (iii) Elevated plasma Hcy increase arterial blood pressures as well as LVEDP and LVSP. The increase in LVSP may reflect the development of hypertension as per an increase in afterload. The large increase in LVEDP reflects a highly significant change in LV preload and/or a decrease in the myocardial index of contractility. The changes seen in LVEDP suggests that elevated plasma Hcy levels may lead to heart failure. Oral heparin treatment reversed the elevation in arterial blood pressure caused by elevated plasma Hcy levels, suggesting its possible role as a treatment for hypertension. Furthermore, LVEDP elevation by elevated plasma Hcy was substantially reduced with oral heparin treatment. This result indicates that oral heparin may prevent the onset of hypertension by protecting the endothelium thus decreasing the LV workload. (iv) There was no clear association of elevated plasma Hcy levels causing increased lipid peroxidation with respect to MDA levels. However, the significant decrease seen in plasma GSH concentrations associated with MD feeding indicates that there is a positive correlation between elevated plasma Hcy and oxidative stress. Oral heparin treatment was seen to attenuate the decrease in GSH levels suggesting that it may have antioxidant activity. (v) Elevated Hcy levels were positively

correlated with increased endothelial cell apoptosis, yet was suppressed by oral heparin treatment.

6.0. REFERENCES

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